Remarks

Reconsideration of this Application is respectfully requested.

According to the Advisory Action, the proposed amendments set forth in Applicants' Amendment and Reply filed on October 21, 2004[1] were not entered. A request for continued examination (RCE) is being submitted concurrently herewith. Therefore, the finality of the Office Action dated July 15, 2004 should be withdrawn and the amendments set forth in Applicants' Amendment and Reply filed on October 21, 2004 should be entered and considered. *See* 37 C.F.R. § 1.114(d).

Upon entry of the amendments set forth in Applicants' Amendment and Reply filed on October 21, 2004, claims 1-6 and 11-16 are pending in the application, with claims 1 and 11 being the independent claims. Claims 7-10 are sought to be canceled without prejudice to or disclaimer of the subject matter therein. Claims 1 and 11 are sought to be amended. No new matter is added by way of these amendments.

Based on the amendments presented in the previous response and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

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[1] In the Advisory Action, the Examiner has referred to "Applicants' amendment and request for reconsideration filed 10/22/2004." Applicants note that the Amendment and Reply Under 37 C.F.R. § 1.116 was filed by Applicants on October 21, 2004. A copy of the date-stamped postcard acknowledging receipt of the Amendment and Reply by the USPTO on October 21, 2004 is attached hereto.

I. Claim Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1-5 and 11-15 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. *See* Office Action, page 2. Applicants respectfully traverse this rejection for the reasons set forth in Applicants' previous replies and for the reasons set forth below.

Even though the amendments set forth in Applicants' Amendment and Reply filed on October 21, 2004 were not entered, the Examiner stated that claims 1 and 11 as amended would be rejected under 35 U.S.C. § 112, first paragraph for lack of enablement and lack of written description. *See* Advisory Action, page 2. As explained below, the enablement and written description requirements of 35 U.S.C. § 112, first paragraph, are fully satisfied for the currently presented claims. Thus, the claims should not be rejected under 35 U.S.C. § 112, first paragraph.

A. The Claims Are Fully Enabled

The currently pending claims have not been rejected for lack of enablement; however, as mentioned above, the Examiner has stated that claims 1 and 11 as amended *would* be rejected for lack of enablement. *See* Advisory Action, page 2. According to the Examiner:

> The specification provides guidance and working examples for the recited method using peptide substrates of SEQ ID NO: 9, SEQ ID

> NO: 11, and SEQ ID NO: 12. However, the specification does not provide enablement for using a peptide comprising the amino acid sequence EXXR as a substrate for separase in the recited method. *Applicants have not clearly shown that any or all of the amino acids in between "E" and "R" (except for those in SEQ ID NO: 9, 11, and 12) can be used as peptide substrates successfully in the claimed method.* Thus, an undue amount of experimentation must be performed to search and screen for any peptide substrate comprising the amino acid sequence EXXR which can be used in the method recited in claims 1 and 11.

See Advisory Action, page 2 (emphasis added). These assertions are insufficient to justify a rejection for lack of enablement.

The Examiner is respectfully reminded that "[i]n order to make a rejection [for lack of enablement], the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention." M.P.E.P. § 2164.04 (citing *In re Wright*, 999 F.2d 1557, 1562, 27 U.S.P.Q.2d 1510, 1513 (Fed. Cir. 1993)). Moreover, "it is incumbent upon the Patent Office, whenever a rejection [for lack of enablement] is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *See* M.P.E.P. § 2164.04 (quoting *In re Marzocchi*, 439 F.2d 220, 224, 169 U.S.P.Q. 367, 370 (CCPA 1971) (emphasis in original)).

There is no evidence on the record to indicate or suggest that making and using the full range of separase substrates comprising an amino acid sequence EXXR would have required undue experimentation. The Examiner has merely stated that "Applicants have not clearly shown that any or all of the amino acids in between "E" and "R" (except for those in SEQ ID NO: 9, 11 and 12) can be used as peptide substrates successfully in the claimed method." As noted above, the M.P.E.P. and the case law make it clear that the Examiner -- *not the Applicants* -- has the initial burden of establishing a reasonable basis to question the enablement provided for the claimed invention. Simply asserting that Applicants have not proven that all peptides having the sequence EXXR would function as separase substrates, is not "acceptable evidence or reasoning" to establish a *prima facie* case of nonenablement.

Not only is there no evidence to support an enablement rejection, but it is also clearly inaccurate, from a technical point of view, that "search[ing] and screen[ing] for any peptide substrate comprising the amino acid sequence EXXR which can be used in the method recited in claims 1 and 11" would have required undue experimentation. There are only twenty different amino acids that each "X" can represent. This amounts to a mere 400 possible EXXR sequences. All 400 possible EXXR sequences are shown on Exhibit 1, submitted herewith. As of the effective filing date of the present application, several EXXR sequences were known in the art to function as separase cleavage sites, including: EVGR; EQGR; ERGR; EYGR; EAGR; and EPSR (found in human and yeast separase cleavage substrates, *see* discussion in *Section B*, below).

In addition, techniques for producing *thousands* of peptides having different amino acid sequences were routine in the art. Such techniques include, *e.g.*, the production of

multiple site-directed DNA mutants that encode the different peptides (*see, e.g.*, Stappert, "Methods for Generating Multiple Site-Directed Mutations *In Vitro*," in *PCR Technology Current Innovations*, CRC Press (1994), copy submitted herewith as Exhibit 2), and the direct production of multiple peptides having specific sequences (*see, e.g.*, Rodda, "Synthesis of Multiple Peptides on Plastic Pins," in *Current Protocols in Protein Science*, John Wiley & Sons, Inc. (1997), copy submitted herewith as Exhibit 3). As noted by Rodda, "The key to preparing large numbers (hundreds to thousands) of synthetic peptides in a short time and at minimal cost is to use a parallel synthesis technique which is efficient and can be done on a small scale." *See* page 18.2.1, first paragraph. Producing separase substrate candidates comprising all 400 of the possible EXXR sequences clearly would have been routine.

Furthermore, the specification teaches methods that can be used to screen numerous peptides for their ability to be cleaved by separase. For example, at page 9, lines 10-20, it is taught that:

> Once the active form(s) for human separase have been obtained by one or more of the methods described above, synthetic peptide substrates for separase are designed and synthesized that allow the simple detection of protease activity in *high throughput format, e.g.*, by fluorogenic methods. The proteolytic assays suitable for this purpose have been described in WO00/486287. By way of example, substrate peptides containing the separase recognition sequence (see WO00/48627) that carry a C-terminal fluorophore

> such as a 7-amino-4-methyl-coumarin group (AMC) *are synthesized by standard methods*. The cleavage of AMC (or other fluorophore groups used) results in a rise in fluorescence which can be measured fluorometrically.

(Emphases added.) Using routine techniques for synthesizing hundreds (if not thousands) of separase substrate candidates, and screening them for the ability to be cleaved by separase using, *e.g.*, a high throughput fluorogenic format as taught in the specification, would have in no way required undue experimentation, especially in view of the numerous EXXR separase substrate cleavage sequences that were known in the art.

The foregoing discussion indicates that routine methods could have easily been employed to make and use the full range of separase substrates used in the practice of the currently claimed methods. In addition, no evidence has been presented to support a rejection for lack of enablement. Accordingly, the claims should not be rejected for lack of enablement.

B. The Claims Are More Than Adequately Described

The Examiner also indicated that the amended claims would be rejected for lack of written description. *See* Advisory Action, page 2. According to the Examiner, the exemplified species of separase substrate peptides disclosed in the specification (SEQ ID NOs: 9, 11 and 12) are not representative of the genus of separase substrates comprising an amino acid sequence EXXR. Applicants respectfully disagree.

The written description requirement for a genus can be satisfied through, *e.g.*, sufficient description of a representative number of species by actual reduction to practice. *See* M.P.E.P. § 2163 (citing *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 1568, 43 U.S.P.Q.2d 1398, 1406 (Fed. Cir. 1997)). What constitutes a "representative number" is an inverse function of the skill and knowledge in the art. *See id.* As indicated above, the level of skill and knowledge in the relevant art was (and is) extremely high. Making numerous peptide substrates and testing them for their ability to serve as proteolytic substrates would have been a matter of routine experimentation. Furthermore, as noted in the specification, "separase cleavage sites in all known organisms cleave after the sequence EXXR." *See* page. 29, lines 18-21 (citing WO 00/48627, Uhlmann *et al.*, *Nature 400*:37-42 (1999); Buonomo *et al.*, *Cell 103*:387-398 (2000); and Hauf *et al.*, *Science 293*:1320-1323 (2001) (copies submitted herewith as Exhibits 4, 5, 6, and 7, respectively)). In addition to the DREIMR sequence of human SCC1, and the SFEILR and EWELLR sequences of human separase (*see* specification at pages 22 and 29), the following EXXR separase cleavage sites were known in the art at the time of the effective filing date of the present application:

1. EVGR (found in *S. cerevisiae* Scc1);
2. EQGR (found in *S. cerevisiae* Scc1);
3. ERGR (found in *S. cerevisiae* Rec8);
4. EYGR (found in *S. cerevisiae* Rec8);
5. EAGR (found in *S. pombe* Rad21);
6. EVGR (found in *S. pombe* Rad21);
7. EVGR (found in *S. pombe* Rec8); and

8. EPSR (found in human SCC1).

See WO 00/48627, page 12, lines 32-34; Uhlmann *et al.*, page 39, Fig. 4b; Buonomo *et al.*, page 388, Fig. 1D; and Hauf *et al.*, page 1321, Fig. 1D. Thus, in view of: (a) the teachings in the specification, (b) the high level of skill in the art, and (c) the numerous EXXR separase cleavage sequences known in the art, the exemplary disclosed species of EXXR separase substrates are clearly representative of the claimed genus. A person of ordinary skill would recognize that, as of the effective filing date of the present application, Applicants were in possession of the complete genus of separase substrates recited in the currently presented claims.

Moreover, the USPTO's own Written Description Guidelines provide further support for Applicants' position that the genus of separase substrates included in the present claims is more than adequately described. For instance, in Example 14 of the Guidelines (copy submitted herewith as Exhibit 8), the written description requirement is found to be satisfied for a claim directed to "A protein having SEQ ID NO: 3 and variants thereof that are at least 95% identical to SEQ ID NO: 3 and catalyze the reaction of A→B." As noted in the Example, the specification describes only one species of the genus (*i.e.*, SEQ ID NO: 3). *See* Written Description Guidelines Example 14, page 54. According to the Guidelines:

> The specification indicates that the genus of proteins that must be variants of SEQ ID NO: 3 does not have substantial variation since all of the variants must possess the specified catalytic activity and must have at least 95% identity to the reference sequence, SEQ ID NO: 3. The single species disclosed is representative of the genus

> because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity.

See id. Thus, the critical factors leading to the conclusion that the claimed subject matter is adequately described are: (1) that the claim includes a structural limitation which limits the variability among species; and (2) that the specification provides an assay for identifying members of the genus that have the recited structural element (at least 95% identical to SEQ ID NO: 3) and the specified functional activity (catalyzing the reaction of A→B).

Both of the above-cited factors are found in the context of the present claims. First, all of the separase substrates included within the present claims must have a particular structural element, namely the amino acid sequence EXXR. This limitation substantially restricts the variability among species; as noted above, there are only 400 possible sequences having the EXXR motif. *See* Exhibit 1. Second, the specification provides an assay for determining if a given peptide having an EXXR sequence is capable of being cleaved by separase. *See, e.g.*, specification at page 9, lines 10-20 (discussing high throughput fluorogenic screening methods that can be used to detect the proteolytic cleavage of substrates). Thus, when the reasoning set forth in Example 14 of the USPTO's Written Description Guidelines is applied to the circumstances surrounding the currently presented claims, it must be concluded that the disclosed species are representative of the genus of EXXR peptide substrates, and therefore the written description requirement is fully satisfied.

Finally, the Examiner's attention is directed to the Federal Circuit's statement in *Regents of the University of California v. Eli Lilly & Co*:

> In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus.

Id., 119 F.3d 1559, 1568 (Fed. Cir. 1997). Here, the generic formula "EXXR" indicates with specificity the genus of separase substrates encompassed by the claims. One skilled in the art can easily distinguish the formula EXXR from other formulae and can easily identify, not just "many," but *all* of the species that are encompassed by this formula. *See* Exhibit 1. Thus, according to the Federal Circuit's statement quoted above, the formula "EXXR" is an adequate description of the claimed genus.

In view of the foregoing, Applicants respectfully submit that the currently presented claims should not be rejected for lack of written description under 35 U.S.C. § 112, first paragraph.

III. Claim Objections

Claims 6 and 16 were objected to as being dependent upon a rejected base claim. *See* Office Action, page 3. In view of the claim amendments set forth in Applicants'

previous response and the comments presented above, Applicants believe that the claim objections are moot and should be withdrawn.

Conclusion

All of the stated grounds of objections and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding objections and rejections and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite allowance of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Supplemental Amendment and Reply is respectfully requested.

Respectfully submitted,

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Date: JUNE 14, 2005

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EXXR Amino Acid Sequences

EIIR	EVIR	EMIR	EAIR	EPIR	ESIR	EWIR	ENIR	EEIR	EKIR
EILR	EVLR	EMLR	EALR	EPLR	ESLR	EWLR	ENLR	EELR	EKLR
EIVR	EVVR	EMVR	EAVR	EPVR	ESVR	EWVR	ENVR	EEVR	EKVR
EIFR	EVFR	EMFR	EAFR	EPFR	ESFR	EWFR	ENFR	EEFR	EKFR
EIMR	EVMR	EMMR	EAMR	EPMR	ESMR	EWMR	ENMR	EEMR	EKMR
EICR	EVCR	EMCR	EACR	EPCR	ESCR	EWCR	ENCR	EECR	EKCR
EIAR	EVAR	EMAR	EAAR	EPAR	ESAR	EWAR	ENAR	EEAR	EKAR
EIGR	EVGR	EMGR	EAGR	EPGR	ESGR	EWGR	ENGR	EEGR	EKGR
EIPR	EVPR	EMPR	EAPR	EPPR	ESPR	EWPR	ENPR	EEPR	EKPR
EITR	EVTR	EMTR	EATR	EPTR	ESTR	EWTR	ENTR	EETR	EKTR
EISR	EVSR	EMSR	EASR	EPSR	ESSR	EWSR	ENSR	EESR	EKSR
EIYR	EVYR	EMYR	EAYR	EPYR	ESYR	EWYR	ENYR	EEYR	EKYR
EIWR	EVWR	EMWR	EAWR	EPWR	ESWR	EWWR	ENWR	EEWR	EKWR
EIQR	EVQR	EMQR	EAQR	EPQR	ESQR	EWQR	ENQR	EEQR	EKQR
EINR	EVNR	EMNR	EANR	EPNR	ESNR	EWNR	ENNR	EENR	EKNR
EIHR	EVHR	EMHR	EAHR	EPHR	ESHR	EWHR	ENHR	EEHR	EKHR
EIER	EVER	EMER	EAER	EPER	ESER	EWER	ENER	EEER	EKER
EIDR	EVDR	EMDR	EADR	EPDR	ESDR	EWDR	ENDR	EEDR	EKDR
EIKR	EVKR	EMKR	EAKR	EPKR	ESKR	EWKR	ENKR	EEKR	EKKR
EIRR	EVRR	EMRR	EARR	EPRR	ESRR	EWRR	ENRR	EERR	EKRR
ELIR	EFIR	ECIR	EGIR	ETIR	EYIR	EQIR	EHIR	EDIR	ERIR
ELLR	EFLR	ECLR	EGLR	ETLR	EYLR	EQLR	EHLR	EDLR	ERLR
ELVR	EFVR	ECVR	EGVR	ETVR	EYVR	EQVR	EHVR	EDVR	ERVR
ELFR	EFFR	ECFR	EGFR	ETFR	EYFR	EQFR	EHFR	EDFR	ERFR
ELMR	EFMR	ECMR	EGMR	ETMR	EYMR	EQMR	EHMR	EDMR	ERMR
ELCR	EFCR	ECCR	EGCR	ETCR	EYCR	EQCR	EHCR	EDCR	ERCR
ELAR	EFAR	ECAR	EGAR	ETAR	EYAR	EQAR	EHAR	EDAR	ERAR
ELGR	EFGR	ECGR	EGGR	ETGR	EYGR	EQGR	EHGR	EDGR	ERGR
ELPR	EFPR	ECPR	EGPR	ETPR	EYPR	EQPR	EHPR	EDPR	ERPR
ELTR	EFTR	ECTR	EGTR	ETTR	EYTR	EQTR	EHTR	EDTR	ERTR
ELSR	EFSR	ECSR	EGSR	ETSR	EYSR	EQSR	EHSR	EDSR	ERSR
ELYR	EFYR	ECYR	EGYR	ETYR	EYYR	EQYR	EHYR	EDYR	ERYR
ELWR	EFWR	ECWR	EGWR	ETWR	EYWR	EQWR	EHWR	EDWR	ERWR
ELQR	EFQR	ECQR	EGQR	ETQR	EYQR	EQQR	EHQR	EDQR	ERQR
ELNR	EFNR	ECNR	EGNR	ETNR	EYNR	EQNR	EHNR	EDNR	ERNR
ELHR	EFHR	ECHR	EGHR	ETHR	EYHR	EQHR	EHHR	EDHR	ERHR
ELER	EFER	ECER	EGER	ETER	EYER	EQER	EHER	EDER	ERER
ELDR	EFDR	ECDR	EGDR	ETDR	EYDR	EQDR	EHDR	EDDR	ERDR
ELKR	EFKR	ECKR	EGKR	ETKR	EYKR	EQKR	EHKR	EDKR	ERKR
ELRR	EFRR	ECRR	EGRR	ETRR	EYRR	EQRR	EHRR	EDRR	ERRR

** The EXXR sequences found in SEQ ID NOs: 9, 11 and 12 are shown in bold.

Chapter 9

METHODS FOR GENERATING MULTIPLE SITE-DIRECTED MUTATIONS *IN VITRO*

Jörg Stappert

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I. INTRODUCTION

A. STRATEGIES

Site-directed mutagenesis of cloned genes as well as the mutation of cis-regulatory elements is a powerful and rapid technique for their functional analysis. Since PCR was first described, a number of PCR-based methods have been developed for introducing directed mutations in virtually any position into DNA or for joining unrelated sequences together.

In the simplest cases, point mutations, deletions or insertions can be engineered at the priming sites, using mismatched primers also covering appropriate restriction sites to facilitate recloning of the PCR product. In many cases however, restriction sites are not available, and many mutations cannot be introduced into various sites along a relatively long DNA fragment. To overcome the problem of having to have suitable restriction sites in the vicinity of the desired mutation, several methods have been developed, based on one of the following strategies:

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1. In the first strategy the entire plasmid is amplified using a pair of primers located back to back (e.g., inverse PCR, IPCR; enzymatic inverse PCR, EIPCR), or two sets of primers are used to amplify two overlapping parts of the plasmid which, after annealing via their short complementary DNA stretches, build up the entire plasmid again (e.g., recombinant circle PCR, RCPCR).
2. The second strategy leads to the amplification of a defined mutagenized fragment, which then has to be recloned. The desired mutations are generated by using two primer sets resulting in overlapping PCR fragments or by joining the mutated primers to a "tagged" primer, which enables the selective amplification of the mutated DNA strand.

Following the concept guidelines of this book, an illustrative summary of some of the currently published techniques will be given, their advantages and disadvantages will be pointed out, and finally, in Section II, protocols for RCPCR and tagged PCR will be described, representing each of the strategies mentioned. For a detailed description of the other methods summarized in this chapter, the reader is referred to the references cited for each procedure.

B. SITE-DIRECTED MUTAGENESIS BY AMPLIFICATION OF THE ENTIRE PLASMID

As mentioned, earlier, to do site-directed mutagenesis of cloned DNA by amplifying the whole plasmid rather than just a fragment, four different methods have been described:

1. Inverse PCR mutagenesis (IPCR)[1]
2. Enzymatic inverse PCR mutagenesis (EIPCR)[2]
3. Recombinant circle PCR (RCPCR)[3]
4. Recombination PCR (RPCR)[4]

These four methods have been derived from the originally published inverse PCR.[5] In this technique, two primers that are located back to back on the opposing DNA strands of a plasmid drive the PCR. The resultant PCR product is a linear DNA molecule identical in length to the starting plasmid. Because all these procedures rely on PCR amplification of the entire plasmid, it is not necessary to prepare an appropriate vector fragment or a single-stranded DNA template.

EIPCR is a technique that combines strategies of inverse PCR with the class 2s restriction site approach of Tomic et al.[6] (see Figure 1A). The key step to EIPCR is the incorporation of identical class 2s restriction sites in the primer set used for PCR. Class 2s restriction enzymes have a recognition site located 5′ of the cut site (e.g., Bsp MI *ACCTGC* NNNN*NNNN*). Thus, after completing PCR, the ends of the full-length linearized plasmid are digested with the class 2s enzyme incorporated into the primers. Due to the distance between recognition and cut site, all sequences upstream of the cut site will be lost. Thus in the ligation the only part that becomes part of the plasmid is the *NNNN* overhang, which can be made to be the native DNA sequence. Mutations can be placed into one or both primers and at any location between the enzyme cut site and the exact 3′ match, which should be of a magnitude >15 bp. Since several 2s restriction enzymes have been described, it should generally be possible to design primers containing an appropriate 2s restriction site. The number of positive clones carrying the desired mutation is more than 95%, which makes EIPCR the most efficient method.

In summary, EIPCR has two major advantages: (1) the high percentage of correct clones and (2) the requirement of only one pair of primers to generate a mutation.

In contrast to EIPCR, two different sets of primers are needed for RCPCR and RPCR.[7] In both methods, the product of one inverse PCR is mixed with the product of a second inverse PCR, which is primed at a different location on the same template. Using RCPCR, these products are combined, denatured, and reannealed *in vitro* before competent *Escherichia coli*

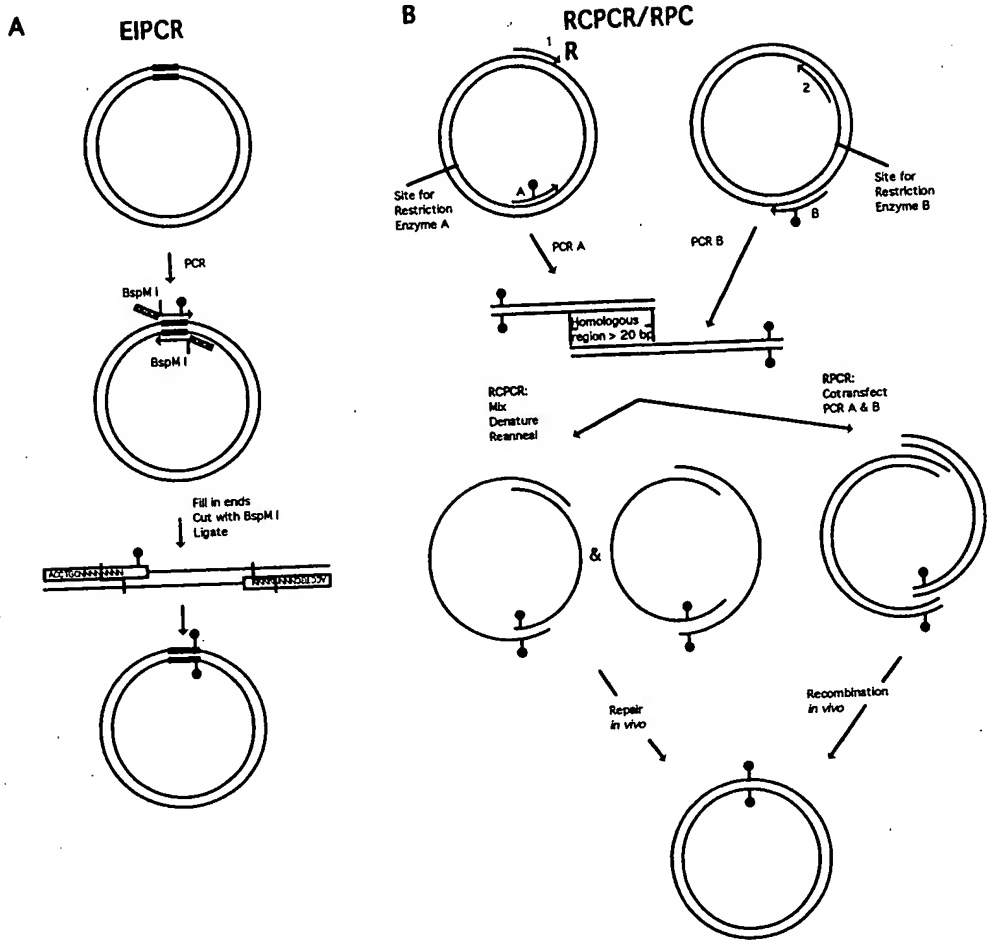

Figure 1. The general scheme for EIPCR (A) and RCPCR/RPCR (B). Double-stranded circles represent double-stranded plasmids; hatched boxes mark 5′ add-on sequences; Solid circles indicate mismatches in the primers and resulting mutations in the PCR products.

are transformed with this construct, as shown in Figure 1B. In contrast to RCPCR, this cross-annealing step is omitted when using RPCR. An equal amount of both PCR reactions is transformed into competent *E. coli*. Upon transformation, the DNA ends undergo homologous recombination *in vivo*, resulting in a circular plasmid. The cross-annealing of the two amplified fragments is mediated by homologous DNA sequences at the 5′ and 3′ and respectively. Whereas the length of homology between mutated ends should be at least 30 bp, the overlapping homology between nonmutated ends depend on the location of primer 1 and 2. Interestingly, it was shown that the number of mutated clones is nearly 100% when using a 22-bp overlap at the nonmutated ends in combination with RPCR, but drops down to 50% when generating the mutation with RCPCR. An inverse correlation was found when increasing the length of homology between nonmutated ends.

All three methods have certain advantages and limitations. All three are rapid and efficient. Nevertheless, the size limit for the template to be amplified is in the range of 5 kb, although mutagenesis of a 7.1-kb construct has been described.[8] A second disadvantage, is the necessity of using relatively long primers; the primers used in these different approaches have to be at least 25 to 30 bp in length. Third, one has to consider the fidelity of the enzyme used for PCR.

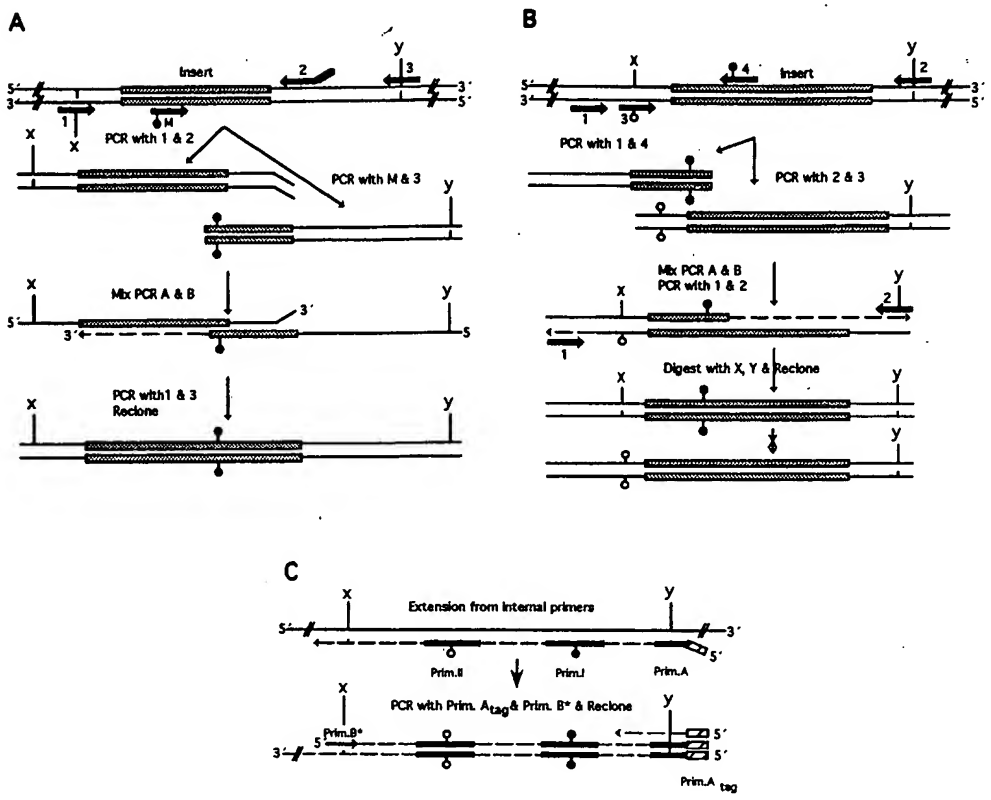

Figure 2. Schematic diagram of three methods used to mutate fragments of a defined length. Primers are represented by black bars; corresponding mismatches are indicated by open and solid circles; Restriction sites are designated X and Y. A detailed description of each method is given in the text.

Since PCR has a low but detectable rate of mutagenesis, polymerases should be used that have a 3′ to 5′ proofreading capability, as this lowers the rate of unwanted mutation.

C. SITE-DIRECTED MUTAGENESIS BY AMPLIFICATION OF SHORT DNA FRAGMENTS

The problem of unwanted PCR errors will be diminished when amplifying only small fragments; this can be achieved by using one of the methods shown in Figure 2.

The two procedures schematically depicted in Figure 2A and 2B represent modifications of the overlap extension method originally published by two groups.[9,10] This method is based on the amplification of two fragments with overlapping ends in which the same mutations are introduced. These fragments are combined and reannealed to each other, and the 3′ overlap of each DNA strand serves as a primer for the 3′ extension of the complementary strand. One disadvantage of the original method is that it requires two new primers for each mutation, limiting one in making an extensive mutagenesis in the same DNA sequence.

The problem has been circumvented by a method described by Mikaelian and Sergeant,[11] as shown in Figure 2A. It requires three universal primers chosen in the vector and only one specific primer for each mutation. As shown, the first step consists of two different rounds of PCR. The two reactions are done using the primer combinations 1,2 and M,3. Primer 1,2 and 3 are homologous to the vector sequence, but primer 2 also contains a mismatched 3′ end; primer M contains the mutation to be introduced into the template DNA. The two amplified fragments are purified, mixed, and subjected to a second PCR with external primers 1 and 3.

During this second PCR, only the mutated DNA strand is amplified, since the 3′ add-on end of primer 2 inhibits the extension of the nonmutated DNA strand. The amplified fragment can be then digested with the appropriate restriction enzymes. The efficiency of this procedure reaches 90%.

A similar approach was used by Ito et al.[12] (Figure 2B). To reclone only the mutated DNA strand, they developed a method called MR (an abbreviation of "modification of a restriction site"). As in the previous technique, three primers are selected as common primers so that each mutation requires only one additional primer. Due to the design of the commonly used primers, it is necessary to reclone the insert to be mutated into a polylinker site of an appropriate plasmid. Even if a suitable restriction site does not exist in the target DNA, it is very easy to create a proper site at both ends of the target DNA by using primers carrying 5′ add-on sequences. As shown in the diagram, primers 1 and 2 are complementary to the neighboring sequences of a polylinker site. Primer 3 is complementary to the polylinker sequence located downstream of primer 1 and has a mismatched nucleotide (nt) to destroy a restriction site. These three primers can be commonly used in a series of different mutagenesis. The fourth primer, however, carries the mutation of interest. By using the primer combinations 1,4 and 2,3, two fragments are amplified, mixed, denatured, and annealed. The resulting products are further amplified by PCR using the external primers 1 and 2, and digested with two different restriction enzymes, X and Y. Although two kinds of DNA are amplified in the second PCR, only the mutated DNA fragment will be recloned, since the restriction site X has been deleted in the nonmutated DNA fragment. Depending on the oligonucleotides used in this technique, the efficiency of getting positive clones can be as high as 100%.

Due to this high efficiency, the described methods are very useful for introducing several mutations into various sites of the inserted DNA. Nevertheless, each new mutation makes it necessary to run a further PCR. Additionally, each newly mutated DNA fragment has to be sequenced in order to detect nucleotide misincorporation generated during DNA amplification.

To avoid this problem, we have developed a method that allows the introduction of several mutations in only one step.[13] To do this we combined extension of mutated internal primers by T4 DNA polymerase with selective amplification of the mutated DNA strand by PCR from added-on external primers. The principle of this method is summarized in Figure 2C.

In the first step, two or even more mutagenic primers (I, II) and Primer A are annealed to the template. Primer A has a suitable restriction site and a 5′ add-on sequence that is not complementary to the template. After primer extension and ligation reaction, the mutated DNA strand is selectively amplified by PCR in the second step by using the two outer primers, A_{tag} and B*, as common primers. Each new mutation thus requires only one additional primer. The percentage of positive clones carrying both mutations depends on whether ss or ds DNA is used as a template. When using ss DNA, up to 80% of all clones will be positive for two mutations, in contrast to 40 to 50% when using ds plasmid DNA. For this method, bacteriophage T4 DNA polymerase or Sequenase should be used in the polymerase extension reaction. Unlike the Klenow fragment of *E. coli* DNA polymerase I, neither of these enzymes is able to displace the mutagenic oligonucleotide from its template. Therefore there should be no limit to the number of different mutagenic primers that can be used in a single *in vitro* site-directed mutagenic reaction. Indeed, as has been reported by Perlack,[15] using the classical procedure of Kunkel,[14] 14% of the clones tested were positive for seven of seven different mutations generated in one single step. This is the same efficiency one would expect when using the PCR-based method.

In the protocols described below, practical approaches concerning the generation of mutations are given exclusively for the mutational step itself. Commonly used techniques like plasmid preparation, ligation, transformation of competent *E. coli*, etc., should be done using accepted protocols.[16,17]

II. MATERIALS AND METHODS

A. MATERIALS

Plasmids used as PCR templates as well as for DNA sequencing were prepared with Qiagen mini- or midi-columns (Qiagen, Düsseldorf). Sequence analysis of the mutants was done on double-stranded DNA, using the T7 Sequenase Kit (U.S. Biochemical) according to the manufacturer's description. All oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and then purified using a C-18 reversed-phase high-pressure liquid chromatography (HPLC) column. Except the *Taq* DNA polymerase, which was purchased from Amersham Buchler (Braunschweig), all restriction and modification enzymes were purchased from GIBCO BRL (Eggenstein) or from Boehringer Mannheim.

B. RECOMBINANT CIRCLE PCR (RCPCR)

1. Primer Design

RCPCR requires a total of four primers per mutagenesis reaction. If the insert to be mutated has been cloned into a polylinker site of a vector, only two new primers need to be synthesized per mutagenesis reaction. Since the nonmutating primers will be located outside the mutagenesis region, they can be reused to mutate any given region of the insert. The nonmutating primers should be at least 15 nt in length, where the mutating primers have an overall length of >25 nt carrying at least 15 nt of exact complementarity to each other at their 5′ ends. It is also possible to use mutating primers with an exact complementarity. The homology length between nonmutated ends will greatly influence the efficiency of this method.

2. PCR Amplification

Before PCR, each plasmid has to be linearized by restriction enzyme digestion outside the region to be amplified. In each of the two separate PCR amplifications for a given mutagenesis, we set up the following reaction mixture in 100 µl: 10 ng of pBlueskript SKII+ DNA containing the insert to be mutated, each primer at 1 µ*M*, 100 µ*M* of each dNTP, 2.5 U *Taq* polymerase in Taq-Buffer (10 × Taq-Buffer is 100 m*M* Tris-HCl, pH 8.4; 500 m*M* NaCl; 20 m*M* $MgCl_2$; 1 mg/ml gelatin). Prior to amplification, 60 µl of mineral oil is placed on top of each reaction mixture. For the amplification, reactants are subjected to 30 c of PCR with the following parameters: denaturation at 95°C for 0.5 min; annealing at 50°C for 0.5 min; and extension at 72°C for 2 min.

3. Purification of the PCR Product

As published by Jones and Winistorfer,[7] there is no need to further purify the PCR products. To remove mineral oil, we recommend freezing the probe. Alternatively, each PCR product may be carefully removed with a thin micropipet and transferred into a new reaction tube.

4. Annealing of the PCR Products

In order to generate recombinant circles *in vitro*, equal amounts of the two PCR products have to be combined, denatured at 95°C for 5 min, reannealed at 50°C for 2 h, and returned to room temperature prior to transfection.

5. Transformation of *E. coli* and Screening of Colonies

Competent *E. coli* should be transformed with 2 to 5 µl of combined PCR product. In cases of restriction site deletions or insertions, positive clones can be identified either by preparation of the plasmid DNA or by using a PCR-based procedure described by Güssow and Clackson.[18] Briefly: Colonies are resuspended in 0.5 ml of water (live bacteria can be rescued at this stage) and boiled in a water bath for 5 min. After centrifugation for 2 min at 13 to 16,000 *g*, 5 µl of the supernatant is subjected to 30 c of PCR amplification with primers that flank the mutagen-

esis site. The orientation of the insert may be screened using a third primer within the insert. Alternatively 1/10 of the unpurified PCR product may be digested by the appropriate restriction enzyme

C. TAGGED PCR MUTAGENESIS METHOD

1. Primer Design

In order to use Primers A and B* together, they should be complementary to vector sequences flanking the insert to be mutated. Primer A should have a length of >30 nt and contain 15 nt at the 5′ end, which are not homologous to the template. A suitable restriction site should be available within the homologous region. Primer A_{tag}, which is identical to the 5′ add-on sequence of primer A, should have a GC content of >50%. Primer B* has to cover a second restriction site and should have a minimum length of 15 nt. The mutagenic primers we use are, respectively, 18 to 21 nt in length and contain up to four mismatches relative to the template DNA. A total of 6 nt exact homology to the template is included on each end to ensure proper hybridization.

2. 5′ Phosphorylation of the Mutating Primers

Before starting primer extension, the mutating primers have to be phosphorylated with Kinase in order to allow ligation of its 5′ ends. Set up the following reaction mixture in 20 µl: 200 pmol primer, 1 × Kinase Buffer (10 × Kinase Buffer is 0.5 *M* Tris-HCl, pH 8.0; 0.1 *M* $MgCl_2$), 10 µ*M* DTT, 2 m*M* ATP, and 5 U polynucleotide kinase. Incubate at 37°C for 30 min and then at 65°C for 10 min to inactivate the kinase. Dilute the phosphorylated oligonucleotides to a final concentration of 2 pmol/µl in water.

3. Denaturation of the ds Template DNA

In order to facilitate annealing of the primers to the template, it is necessary to denature the ds DNA. This can be done either by heat or alkaline denaturation. In both procedures, use 0.2 to 0.4 pmol ds template DNA. If using alkaline denaturation, incubate the template DNA in a reaction volume of 20 µl, together with 0.2 *M* NaOH, at room temperature for 5 min. Neutralize the mixture by adding 8 µl 5 *M* ammonium acetate, pH 7.4. Precipitate the DNA with 100 µl ethanol at –70°C for 5 min. Centrifuge at 16,000 *g* for 5 min, then wash with 70% ethanol. Dry at room temperature for 10 min.

4. Annealing and Extension Reaction

To anneal primers to the denatured template DNA, incubate 5 to 10 × excess of phosphorylated primers (mutagenized primers and primer A) in 1 × Annealing Buffer (10 × Annealing Buffer is 200 m*M* Tris-HCl, pH 7.4; 20 m*M* $MgCl_2$; 500 m*M* NaCl) in a total volume of 10 µl. Heat to 65°C for 3 min, and allow to cool slowly to room temperature for 30 min.

Synthesis of the complementary DNA strand is done in a volume of 20 µl containing the same annealing mixture plus 2.5 U T4 DNA polymerase, 1 U T4 DNA ligase, and 1 × Synthesis Buffer (10 × Synthesis Buffer is 5 m*M* of each dNTP; 10 m*M* ATP; 100 m*M* Tris-HCl, pH 8.0; 10 m*M* EDTA). After incubating at 37°C for 90 min, add 10 m*M* Tris-HCl; 10 m*M* EDTA, pH 8.0 (final concentration), and stop the reaction by freezing.

5. PCR Amplification

For amplification of the mutated DNA strand use the external primers, A_{tag} and B*, at 1 µ*M* with 1/5 volume of the crude "extension" reaction, 100 µ*M* of each dNTP, and 2.5 U *Taq* polymerase in Taq-Buffer (for 10 × Taq-Buffer, see RCPCR) in a reaction volume of 100 µl. Before amplification, overlay the mixture with 60 µl of mineral oil. Then subject the PCR mixture to 30 c of amplification: 0.5 min at 95°C; 0.5 min at 55°C; 1 min at 72°C. After removing the mineral oil, 5 µl of the PCR mixture can be analyzed on a 1% TBE agarose gel

and stained with EtBr. To determine the efficiency of incorporation of the mutated primers, 5 µl of each reaction should be digested with the appropriate restriction enzymes. Before recloning, we recommend purifying the PCR products. Clones can be analyzed as described above.

III. DISCUSSION

As mentioned above, the efficiency of the RCPCR method is influenced by the length of homology between nonmutated ends. Whereas 50% of all clones are positive when containing a 25-bp stretch of homology, up to 90% of the clones will have the mutation if the length of homology is increased to 2800 bp. In order to reduce background transformations by the original nonmutated PCR template, one must use linearized PCR templates, which have been cut outside the region to be amplified.

One big advantage of RCPCR, as well as of all other methods using the mutated primers to amplify the entire plasmid for amplification, is the fact that cloning steps are omitted. Nevertheless, for each mutation it is necessary to do a new round of amplification. Site-directed mutagenesis using the tagged PCR method is therefore the method of choice if several mutations have to be generated.

The possibility of simultaneously introducing several mutations in a single step makes this method much faster than the others as long as suitable "markers" can be introduced in each mutation. For that reason it is necessary to use mutagenic oligonucleotides carrying the desired mutation together with a second nt mismatch. This second, "silent" mutation is necessary to generate or delete a restriction site within the primer sequence in order to facilitate screening for positive clones. If such markers are absent, the method becomes more elaborate because all colonies have to be hybridized using the primers as probes. On the other hand, this problem will still occur in all methods not approaching a mutation efficiency of 100%.

For this reason, it is necessary in each case to choose the *in vitro* mutagenesis method appropriate for a given problem.

REFERENCES

1. **Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G., and Galas, D. J.,** A simple method for site-directed mutagenesis using the polymerase chain reaction, *Nucleic Acids Res.*, 17, 6545, 1989.
2. **Stemmer, W. and Morris, K. M.,** Enzymatic inverse PCR: a restriction site independent, single-fragment method for high-efficiency, site-directed mutagenesis, *BioTechniques*, 13, 215, 1992.
3. **Jones, D. H. and Howard, B. H.,** A rapid method for site-specific mutagenesis and directional subcloning by using the polymerase chain reaction to generate recombinant circles, *BioTechniques*, 8, 178, 1990.
4. **Jones, D. H. and Howard, B. H.,** A rapid method for recombination and site-specific mutagenesis by placing homologous ends on DNA using polymerase chain reaction, *BioTechniques*, 10, 62, 1991.
5. **Ochman, H., Gerber, A. S., and Hartl, D. L.,** Genetic applications of an inverse polymerase chain reaction, *Genetics*, 120, 621, 1988.
6. **Tomic, M., Sunjevaric, I., Savtchenko, E. S., and Blumenberg, M.,** A rapid and simple method for introducing specific mutations into any position of DNA leaving all other positions unaltered, *Nucleic Acids Res.*, 18, 1656, 1990.
7. **Jones, D. H. and Winistorfer, S. C.,** Recombinant circle PCR and recombination PCR for site-specific mutagenesis without PCR product purification, *BioTechniques*, 12, 528, 1992.
8. **Yao, Z., Jones, D. H., and Grose, C.,** Site-directed mutagenesis of herpesvirus glycoprotein phosphorylation sites by recombination polymerase chain reaction, *Methods Applic.*, 1, 205, 1992.
9. **Higuchi, R., Krummel, B., and Saiki, R. K.,** A general method for *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions, *Nucleic Acids Res.*, 16, 7351, 1988.
10. **Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R.,** Site-directed mutagenesis by overlap extension using the polymerase chain reaction, *Gene*, 77, 51, 1989.
11. **Mikaelian, I. and Sergeant, A.,** A general and fast method to generate multiple site directed mutations, *Nucleic Acids Res.*, 20, 376, 1992.

12. **Ito, W., Ishiguro, H., and Kurosawa, Y.,** A general method for introducing a series of mutations into cloned DNA using the polymerase chain reaction, *Gene*, 102, 67, 1991.
13. **Stappert, J., Wirsching, J., and Kemler, R.,** A PCR method for introducing mutations into cloned DNA by joining internal primer to a tagged flanking primer, *Nucleic Acids Res.*, 20, 624, 1992.
14. **Kunke, T. A.,** Rapid and efficient site-specific mutagenesis without phenotype selection, *Proc. Natl. Acad. Sci. U.S.A.*, 82, 488, 1985.
15. **Perlack, F. J.,** Single step large scale site-directed *in vitro* mutagenesis using multiple oligonucleotides, *Nucleic Acids Res.*, 18, 7457, 1990.
16. **Maniatis, T.,** *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
17. **Ansubel, F. M.,** *Current Protocols in Molecular Biology*, John Wiley & Sons, New York.
18. **Güssow, D. and Clackson, T.,** Direct clone characterization from plaques and colonies by the polymerase chain reaction, *Nucleic Acids Res.*, 10, 4000, 1989.

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Synthesis of Multiple Peptides on Plastic Pins

Scanning protein sequences by bioassay for smaller bioactive peptide sequences requires a source of many peptides homologous with the parent protein sequence. This unit deals with one of the synthetic methods for making such sets of peptides (see Fig. 18.2.1). The key to preparing large numbers (hundreds to thousands) of synthetic peptides in a short time and at minimal cost is to use a parallel synthesis technique which is efficient and can be done on a small scale. The multipin technology is suitable because it can be performed without expensive synthesizers and it uses equipment available to most laboratories. Prior experience with organic synthesis techniques or peptide chemistry is useful but not essential. The products of synthesis by multipin technology are unpurified peptides which are useful as screening reagents and may also be used to prepare purified peptide on a small scale.

Most multipin techniques exploit the conventional 8 × 12 matrix layout of common microtiter equipment which simplifies handling of the synthesis, the products (peptides), and the test results. Computer assistance with synthesis and data analysis also speeds the cycle from designing the experiment through analyzing the results.

With multipin technology, peptides are synthesized in parallel on plastic "pins" (Fig. 18.2.2) to give sets of peptides suitable not only for B and T cell epitope scanning but also for other bioassays. Peptides can be either permanently bound to the surface of the plastic for direct binding assays, or they can be released into solution. There is a choice of N- and C-terminal peptide endings. For solution-phase peptides, the synthesis scale can be 1 or 5 μmol (for a 10-mer, ~1 mg or 5 mg, respectively). The preferred coupling/deprotection chemistry used is the milder 9-fluorenylmethyloxycarbonyl (Fmoc) protection scheme rather than the older *t*-butyloxycarbonyl (*t*-Boc) protection scheme (see *UNIT 18.1*), thus reducing the level of chemical safety risk arising from synthetic peptide chemistry.

This unit covers the strategy of the multiple peptide approach to biological scanning, the synthetic protocols, and the handling of peptides after synthesis—cleavage, preliminary purification, storage, and analysis (see Basic Protocol). It is specific for the multipin technique using equipment obtained from Chiron Technologies, although some of the approaches are applicable to other multiple synthesis techniques. Procedures for multipin equipment obtained from other suppliers may differ from the procedures described here, and the manufacturer's literature should be consulted. This unit also includes protocols for preparing Fmoc–amino acid solutions (see Support Protocol 1) and for acetylating (see Support Protocol 2) or biotinylating (see Support Protocol 3) synthesized peptides.

STRATEGIC PLANNING

For a protein whose primary structure is known, the conceptually simplest method of locating all the bioactive linear peptide sequences is to make all possible peptide subsets of the protein sequence and test them. If only selected parts of the sequence are synthesized, or only the predicted active parts, bioactive sequences could be missed. The use of a set of highly overlapping peptides likewise reduces the possibility that the most bioactive sequences might be missed because they are absent from the set. A set of all overlapping 20-mers offset along the sequence by one residue at a time should capture the entire set of, for example, helper T cell epitopes, and this is a much more reliable approach than trying to predict motifs. In reality, a synthetic peptide scan through a protein is a compromise between the cost and effort in making and screening all peptides and the

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need for completeness. Thus, one worker may choose to make all overlapping 8-mers to find the linear (continuous) B cell epitopes, and another may make 12-mers offset along the sequence by five residues for the same purpose. In each case, all sequences of eight residues from the protein are present in at least one peptide, but the latter approach requires only one-fifth the number of peptides.

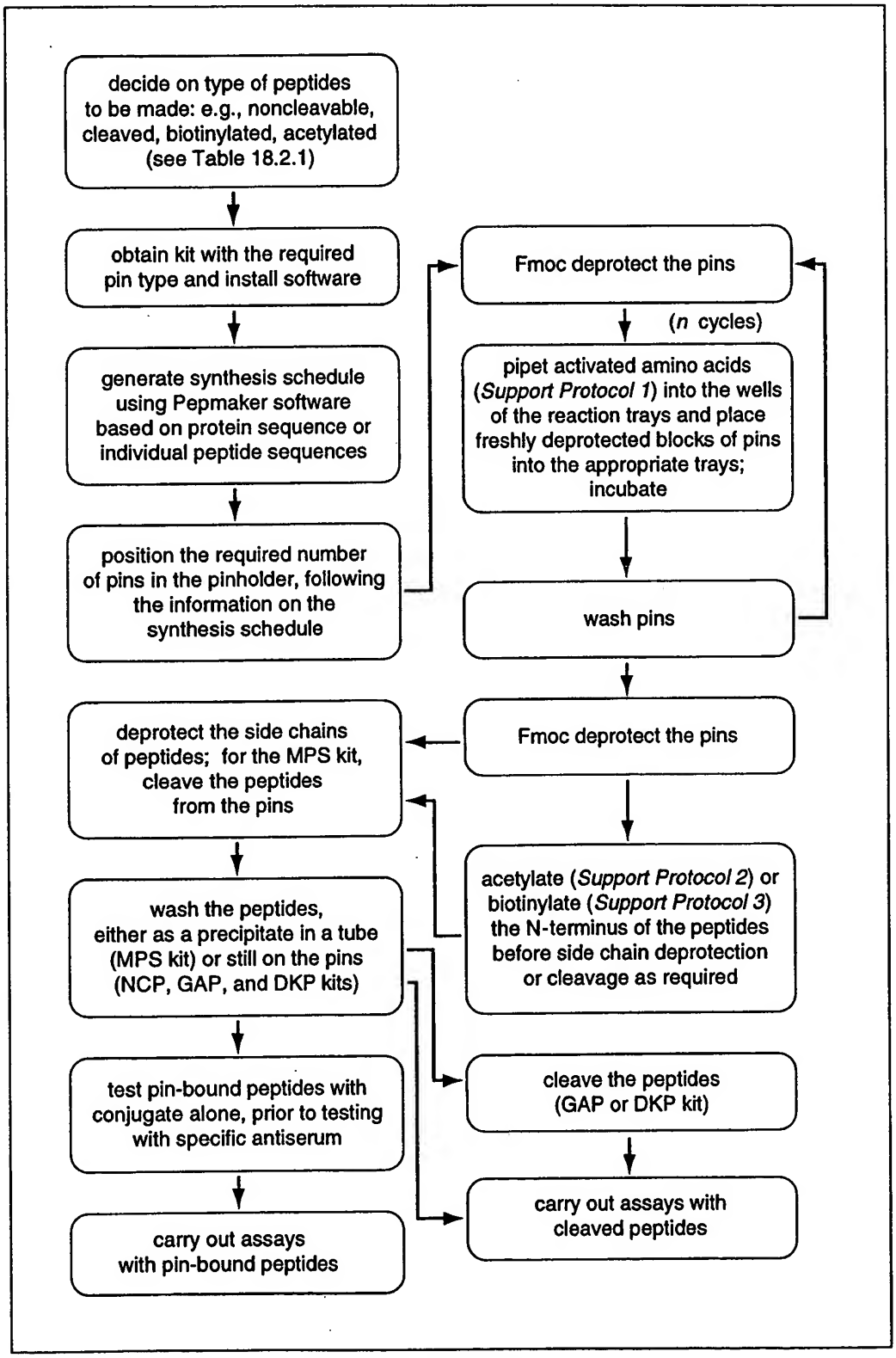

Figure 18.2.1 Flow chart for multipin peptide synthesis.

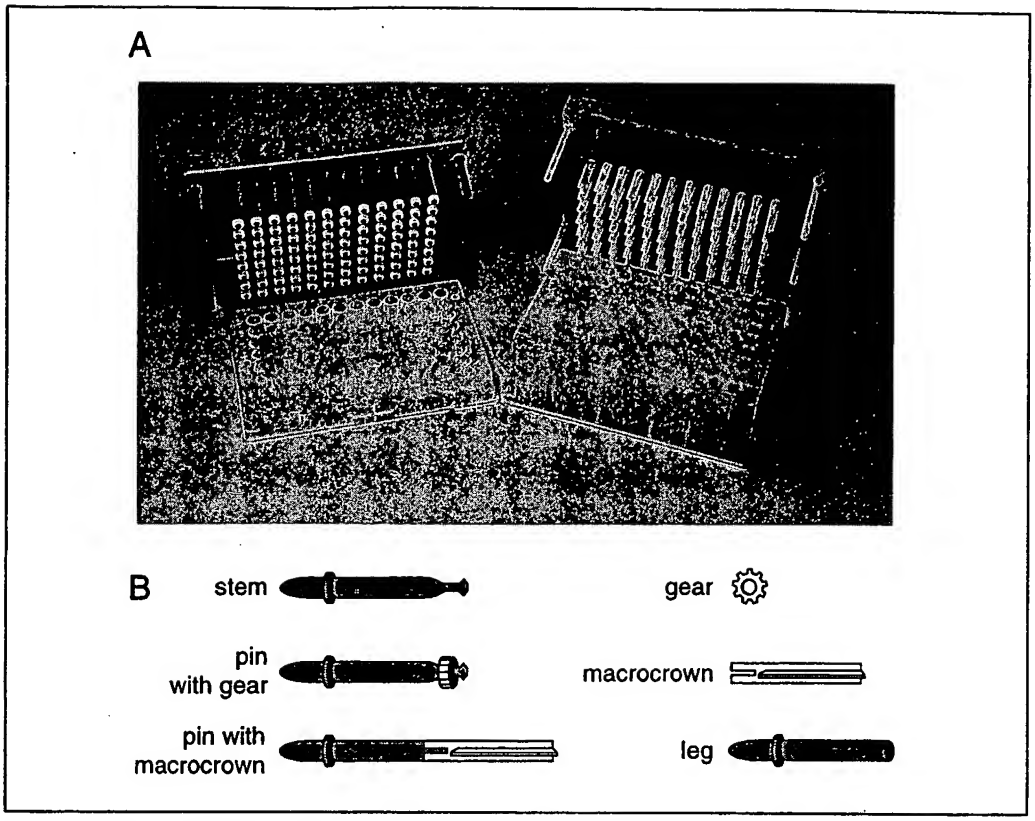

Figure 18.2.2 Apparatus for multipin peptide synthesis. (**A**) Assembled synthesis block with 96 gears (left) or 96 macrocrowns (right). (**B**) Components of the pin assembly. Components are either push-fitted together (e.g., legs or stems into the pin holder) or clipped on (gears or macrocrowns onto stems). All components are solvent-resistant plastic, either polyethylene, polypropylene, or copolymers of these two monomer types.

Planning the Synthesis

Synthetic peptides are assembled by solid-phase synthesis one amino acid at a time, commencing with the C-terminal end of the peptide on the solid phase (see *UNIT 18.1*).

The assembly process, or coupling, requires activation of the α-carboxyl group of each incoming amino acid to make it reactive with the α-amino group of the growing peptide chain. To prevent unwanted polymerization or side reaction, reactive groups in each amino acid must be temporarily protected, and the protecting group removed before further reaction can be carried out. The protecting group on the α-amino function of the most recently added amino acid must be removed before another amino acid can be coupled to it, so the α-amino protection must be labile under conditions that do not remove side-chain protection. Later, the side-chain-protecting groups must be removable under conditions that do not attack the peptide bonds. The two common protecting group "schemes" are known as *t*-butoxycarbonyl (*t*-Boc) or 9-fluorenylmethyloxycarbonyl (Fmoc). The protecting group scheme currently recommended for multipin peptide synthesis is the milder Fmoc scheme, which is the only scheme described in this chapter.

Before beginning to plan the actual synthesis in detail, a choice needs to be made regarding how the peptides will eventually be presented in the bioassay. The options available to investigators are listed in Table 18.2.1.

For noncleavable peptide (NCP) kits, peptides are permanently bound on the solid phase (pin surface) and can be used for direct binding assays but not for interaction with living cells or other complex (e.g., multicomponent) systems. In this case, the peptides must be

Table 18.2.1 Types of Pins for Multipin Peptide Synthesis[a]

Name	Linker[b]	Physical format[c]	Loading	Final form of peptide
NCP	Noncleavable	Gear	50 nmol	(N-capping)-PEPTIDE-linker-pin
MPS	AA ester	Macrocrown	5 μmol	(N-capping)-PEPTIDE-acid
MPS	Rink amide	Macrocrown	5 μmol	(N-capping)-PEPTIDE-amide
DKP	DKP-forming	Gear	1 μmol	(N-capping)-PEPTIDE-DKP
GAP	Glycine ester	Gear	1 μmol	(N-capping)-PEPTIDE-glycine-acid

[a]Abbreviations: DKP, diketopiperazine; GAP, glycine acid peptide; MPS, multiple peptide synthesis; NCP, noncleavable peptide; (N-capping), a free amine, acetyl group, or biotin; PEPTIDE, the sequence of the peptide being made.

[b]Nature of linker between peptide and graft polymer on the pin: noncleavable linker, β-alanine-hexamethylenediamine; DKP, diketopiperazine; AA ester, amino acid ester; Rink amide, Rink amide–forming linker.

[c]See Figure 18.2.2B.

"regenerated" between repeat assays by disrupting the peptide-ligand interaction without damaging the peptide. The quantity of peptide made is very small (50 nmol), but it is sufficient to provide a high surface density of peptide for direct binding assays.

In the other options, peptides are synthesized on pins and then released into solution. The mechanism of peptide release into solution affects the postsynthesis handling and thus the suitability of peptides produced by each cleavage method for various assay systems.

For multiple peptide synthesis (MPS) kits, the released peptides have a "native" free acid or an amide carboxy terminus. To make free acid C-termini, it is necessary to use macrocrowns that already have the first (C-terminal) amino acid on them because the chemistry of forming the first (ester) link is too difficult for the inexperienced user. In contrast, the Rink amide linker allows formation of a peptide with a C-terminal amide of any amino acid by adding the C-terminal amino acid to the Rink handle macrocrown using the standard amino acid coupling protocol. A Rink amide linker is a linker that can accept an amino acid but then can be cleaved in trifluoroacetic acid (TFA) to release the amide form of that amino acid (Rink, 1987). Although acid or amine endings are often the most desirable peptide format to have, they are also the most complex to produce because the cleavage of the peptides from the pin is into neat TFA plus scavengers which needs to be evaporated to recover the peptide. The scale of peptide synthesis for MPS kits is 5 μmol (~5 mg of a decamer).

For glycine acid peptide (GAP) kits, peptides with a glycine at the carboxy terminus are cleaved as the free acid, so that the C-terminal residue is a natural amino acid (glycine) and is not blocked. The peptides are also relatively simple to release from the pin and require little postsynthesis handling. However, the presence of glycine at the C-terminus may be undesirable where the C-terminus plays an important role in peptide bioactivity. The scale of synthesis for GAP kits is 1 μmol (~1 mg of a decamer).

In diketopiperazine (DKP) kits, peptides are synthesized with a DKP group at the C terminus. The DKP group is a cyclic dipeptide formed from C-terminal lysine and proline residues during the facile cleavage of the peptide under the mildest possible conditions: neutral aqueous buffer. In applications where the presence of the DKP group is acceptable, this type of peptide can make the downstream processing of synthetic peptides very simple and fast. The peptides can be placed into a bioassay system immediately after completing the cleavage. The scale of synthesis for DKP kits is 1 μmol (~1 mg of a decamer).

For these five kit options, it is also possible to choose a variety of N-terminal endings on the peptides. For example, it may be desirable to acetylate pin-bound peptides (see Support Protocol 2) to eliminate the positive charge that would otherwise be present on

the α-amino group of the N-terminal residue, or to enhance the activity of a peptide in a T helper assay (Mutch et al., 1991). A handy option for cleaved peptides is to place a biotin group on the N-terminus (see Support Protocol 3) so the peptide can be captured using avidin or streptavidin. These additions must be made prior to side-chain deprotection of the peptides.

There are other configurations for multiple peptide synthesis—e.g., the SPOTS or "peptides on paper" system (Zeneca/CRB), the RaMPS system (DuPont), and multi-synthesizer machines (e.g., Advanced ChemTech).

Assessing Peptide Sequences

Peptides differ so much in properties that it is important to assess the likely properties of the peptides before attempting to synthesize them. Peptide length and hydrophobicity are the two main attributes affecting successful synthesis. The longer the peptide, the lower will be the purity of the product, as each amino acid coupling cycle is never 100% efficient. Synthesis of peptides longer than 20 residues should be avoided unless special attention can be given to each sequence. Hydrophobic peptides may be difficult to synthesize, but more significantly they may be poorly soluble in aqueous buffers, restricting their ultimate usefulness in bioassays. Prior to beginning synthesis of a set of peptides, it is sensible to assess them all for hydrophobicity (Fauchere and Pliska, 1983; *UNIT 2.2*) and decide if all should be attempted as they stand. In many cases, it is possible to choose slightly different peptides (longer, shorter, or using a different starting and finishing point in the homologous protein sequence) that will have more user-friendly properties.

As well as these general factors affecting peptides, particular peptide sequences may have characteristics that make them difficult to synthesize, or they may be problematic after synthesis. It is not feasible to discuss all the common problems here. To help assessment of peptide sequences, a software application called Pinsoft is available free from Chiron Technologies. This allows any sequence to be typed in, and an assessment is automatically reported.

Generating Peptide Sequences

Computer software (Pepmaker) supplied with synthesis kits allows sets of overlapping peptide sequences to be generated from a protein sequence computer file using the single-letter amino acid code. Alternatively, sequences can be created using a word processor and the resulting computer text file can then be used by Pepmaker to guide synthesis. The use of this software simplifies the otherwise complex and tedious task of adding the right amino acids to each reaction plate on each synthesis cycle.

MULTIPIN SYNTHESIS OF PEPTIDES

BASIC PROTOCOL

Derivatized pins with macrocrowns or gears are attached to a pin holder. Each peptide is built up on the reactive surface of one pin by successive cycles of amino acid coupling, followed by washing and removal of the 9-fluorenylmethyloxycarbonyl (Fmoc) amino-protecting group to prepare for the next amino acid coupling cycle. A critical step is properly dispensing activated amino acid solutions into the appropriate wells of each reaction tray. A list of the well locations for dispensing of each amino acid is generated by the Pepmaker software for this purpose. When the peptides are complete, trifluoroacetic acid (TFA) that contains scavengers is used to remove the side-chain-protecting groups, and for MPS kits, to cleave the peptides from the pins. The manual provided with each type of kit (see Table 18.2.1) includes instructions and hints for kit-specific procedures.

NOTE: All reagents should be of the highest grade possible, preferably peptide synthesis or analytical reagent grade.

Materials

20% (v/v) piperidine/dimethylformamide (DMF; see recipe)
DMF, analytical reagent grade
Methanol, analytical reagent grade
100 mM activated 9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acid solutions (see Support Protocol 1)
Side chain deprotecting (SCD) solution (see recipe)
Acidified methanol: 0.5% (v/v) glacial acetic acid/methanol
1:2:0.003 (v/v/v) ether/petroleum ether/2-mercaptoethanol (2-ME)
1:2 (v/v) ether/petroleum ether
0.1 M NaOH
0.1 M acetic acid
0.1 M sodium phosphate buffer, pH 8.0 (*APPENDIX 2E*)
Sonication buffer (see recipe)

Peptide Synthesis Starter Kit (e.g., Chiron Technologies) of the desired type, containing:
  Pepmaker computer program and ELISA reading and plotting programs
  Manual
  Pins with gears or macrocrowns
Storage boxes or sealable bags, polyethylene or polypropylene (ICN Biomedicals)
Pipettor tips, polyethylene or polypropylene (ICN Biomedicals)
0.3- or 1.5-ml reaction trays, polyethylene or polypropylene (Chiron Technologies, Nunc, or Beckman)
Sonicator with power output of ~500 W
Dry nitrogen
Rack containing 96 1-ml polypropylene tubes (Bio-Rad)
10-ml capped conical polypropylene centrifuge tubes

Additional reagents and equipment for N-terminal acetylation (see Support Protocol 2; optional) or biotinylation (see Support Protocol 3; optional)

CAUTION: Perform all chemistry steps in a well-functioning chemical fume hood. Wear solvent-resistant gloves, safety glasses, and protective clothing. The reagents can be flammable, toxic, and/or carcinogenic. Avoid sources of contamination which may affect the pins, including direct contact with the bench surface or exposure to vapors. The reagents for multipin synthesis can be handled in unsealed systems, but the amount of time that these reagents are left exposed to the open air should be minimized by using capped containers for liquids or polyethylene bags for pins wherever practical. Local regulations for safe disposal of solvents and used reagents must be followed.

Prepare synthesis schedule and equipment

1. Use the Pepmaker computer program according to the instructions to generate the required set of peptide sequences (Fig. 18.2.3). Generate the printouts, which show for each coupling cycle how much of each amino acid solution, catalyst, and activating agent needs to be prepared (see Fig. 18.2.4) and where each amino acid solution is to be added to the reaction tray (see Fig. 18.2.5).

   The standard microtiter plate layout is an 8 × 12 matrix, in which the eight rows are identified as A through H and the twelve columns are identified as 1 through 12. However, the Pepmaker software uses a designation in which the column number is given first followed by a number designation for the row, beginning with row H, given in parentheses—i.e., 1(1) for well H1, 1(2) for well G1, 2(1) for well H2, and 12(8) for well A12 (see Fig. 18.2.6).

2. Label each pin holder block indelibly on the top (e.g., Synthesis #1, Block A, Synthesis #1, Block B, and so forth), preferably by scratching into the plastic with a sharp tool. Place the label where it will help orient the block so that the pins are not accidentally placed into amino acid solutions in an inverted orientation. For example, keep pin H1 and well H1 at the lower left corner of the block (Fig. 18.2.6).

   Ink labels will run or disappear with exposure to solvents.

   The multipin system is based on the standard microtiter plate layout. The ***block*** *is the complete unit and consists of the* ***pin holder****, which is the support that holds 96* ***pins*** *(in an 8 × 12 array with standard ELISA microtiter plate spacing), and five* ***legs*** *to support the device and correctly position the active surfaces. A pin consists of an inert* ***stem*** *that supports either a* ***gear*** *or a* ***macrocrown****, both of which have an active surface on which the peptide is synthesized (see Figure 18.2.2). A gear is a detachable gear-shaped unit that fits on the thin end of a stem. A macrocrown is a detachable, vaned tip that fits on the thin end of a stem. It is made of high-density polyethylene and the surface is derivatized to give a solvent-compatible polymer matrix. Macrocrowns are provided in two forms: one has a linker that cleaves to give peptides with an amide at the carboxy terminus; the other has a linker that cleaves to give the free acid at the carboxy terminus and is supplied with an amino acid already attached to the linker. The* ***reaction tray*** *used for the synthesis is a polyethylene or polypropylene tray consisting of 96 wells in the standard microtiter plate 8 × 12 matrix. Shallow reaction trays (0.3-ml) are used with gears and deep trays (1.5-ml) are used with macrocrowns.*

3. Remove any pins that are not required for the first cycle of amino acid coupling and store them dry in a plastic bag in the refrigerator until needed.

   Some pins need to be removed when the peptides in the synthesis are of various lengths because the software is designed to arrange all peptides to complete their synthesis on the same (final) coupling cycle. This approach eliminates unnecessary Fmoc–deprotection cycles for pins that are designated to carry the shorter peptides. The synthesis printout from the Pepmaker software shows which pins need to be added for each cycle of amino acid addition (Fig. 18.2.5). Pins (stems) can be pushed out from the top side of the pin holder. In the case of the MPS kit, where the first amino acid is already on the macrocrown as supplied, choose and mount the correct macrocrown for each position on the block.

Deprotect α-amino groups

4. Add 20% piperidine/DMF to a bath and place the pins in the bath so that the tips (macrocrowns or gears) are covered. Cover and let stand for 20 min at room temperature.

   CAUTION: Piperidine is flammable.

   The volume of reagent needed for all the bath steps depends on the shape of the bath, the critical factor being that all surfaces of the pins (i.e., the gears or macrocrowns) bearing the peptide need to be totally covered. A small bath suitable for gears is the upturned polypropylene lid of a pipettor tip box. For macrocrowns, deeper baths or deep-well polypropylene trays as supplied with the kit can be used.

5. Remove the block from the bath, shake off the excess liquid, and then wash the pins in a DMF bath for 2 min at room temperature.

   Again, the DMF must fully cover the tips.

6. Shake off the excess DMF and immerse the block completely in a deep bath of methanol for 2 min so that all surfaces of the block are washed.

   CAUTION: Methanol is flammable and toxic.

   In a shallower bath the block can be turned over so that the pin holder part is washed as well.

```
A      GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1          Page 1
  Description: Example of a scan through Sperm Whale Myoglobin
         8-mer peptides based on the sequence MBN-SW
               Peptide spacing increment is 1
      Segment  1:  146 peptides starting at residue 1
           First peptide: [VLSEGEWQ]
           Last  peptide: [YKELGYQG]
           Protein sequence: MBN-SW (153 residues)
    1: VLSEGEWQLV  LHVWAKVEAD  VAGHGQDILI  RLFKSHPETL  EKFDRFKHLK
   51: TEAEMKASED  LKKHGVTVLT  ALGAILKKKG  HHEAELKPLA  QSHATKHKIP
  101: IKYLEFISEA  IIHVLHSRHP  GNFGADAQGA  MNKALELFRK  DIAAKYKELG
  151: YQG
               Amino Acid set to be used - AASET1
    aaset 1:Free acid L-Fmoc amino acids - DIC/HOBt chemistry
  Number of copies of each peptide  1
  Schedule based on a 250 microliter fill/well
    (Well concentration is 100 mM)
```

Figure 18.2.3 *(above and at right)* A portion of the synthesis schedule worksheets generated by Pepmaker software for Schedule no. 1 for synthesis of a set of all possible overlapping octamers of sperm whale myoglobin. (**A**) This page of the synthesis schedule is a summary of the features of the protein including its sequence. (**B**) This page of the synthesis schedule shows the sequences of the first 96 peptides that will be synthesized, the first two of which are the controls, PLAQGGGG and GLAQGGGG. Peptide sequences are shown in the conventional amino-to-carboxy-terminal direction (from left to right), with a "<" sign indicating the end attached to the solid phase during synthesis. Because amino acid couplings are carried out in the carboxy-to-amino direction, the first amino acids coupled are at the right-hand end of each sequence, adjacent to the "<".

7. Place the block in a second methanol bath to fully cover the tips. Wash for 2 min. Repeat this washing step again with a fresh methanol bath for a total of three methanol washes.

8. Remove the block and allow it to air dry in an acid-free fume hood for a minimum of 30 min.

   Avoid exposure to acidic fumes as this could prevent efficient coupling in the next step.

   The block can be conveniently left to dry while the amino acid solutions are being dispensed.

Dispense activated amino acid solutions

9. Dispense the required volume of each activated amino acid solution (see Support Protocol 1; 150 µl for gears or 450 µl for macrocrowns) into the appropriate wells of the reaction tray as specified by the synthesis schedule for the coupling cycle (e.g., Fig. 18.2.5).

Perform the amino acid coupling

10. Place the pins in the activated amino acid solutions in the reaction tray, ensuring that the block is correctly oriented before actually lowering the pins into the solution. Incubate ≥2 hr at 20° to 25°C in a polyethylene box with a lid or in a sealable polyethylene bag.

    Coupling begins immediately and is irreversible.

B GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1 Page 2

Amino terminus is printed on the left

1	A 1(1)PLAQGGGG<	49	A 1(5)KHLKTEAE<
2	A 2(1)GLAQGGGG<	50	A 2(5)HLKTEAEM<
3	A 3(1)VLSEGEWQ<	51	A 3(5)LKTEAEMK<
4	A 4(1)LSEGEWQL<	52	A 4(5)KTEAEMKA<
5	A 5(1)SEGEWQLV<	53	A 5(5)TEAEMKAS<
6	A 6(1)EGEWQLVL<	54	A 6(5)EAEMKASE<
7	A 7(1)GEWQLVLH<	55	A 7(5)AEMKASED<
8	A 8(1)EWQLVLHV<	56	A 8(5)EMKASEDL<
9	A 9(1)WQLVLHVW<	57	A 9(5)MKASEDLK<
10	A10(1)QLVLHVWA<	58	A10(5)KASEDLKK<
11	A11(1)LVLHVWAK<	59	A11(5)ASEDLKKH<
12	A12(1)VLHVWAKV<	60	A12(5)SEDLKKHG<
13	A 1(2)LHVWAKVE<	61	A 1(6)EDLKKHGV<
14	A 2(2)HVWAKVEA<	62	A 2(6)DLKKHGVT<
15	A 3(2)VWAKVEAD<	63	A 3(6)LKKHGVTV<
16	A 4(2)WAKVEADV<	64	A 4(6)KKHGVTVL<
17	A 5(2)AKVEADVA<	65	A 5(6)KHGVTVLT<
18	A 6(2)KVEADVAG<	66	A 6(6)HGVTVLTA<
19	A 7(2)VEADVAGH<	67	A 7(6)GVTVLTAL<
20	A 8(2)EADVAGHG<	68	A 8(6)VTVLTALG<
21	A 9(2)ADVAGHGQ<	69	A 9(6)TVLTALGA<
22	A10(2)DVAGHGQD<	70	A10(6)VLTALGAI<
23	A11(2)VAGHGQDI<	71	A11(6)LTALGAIL<
24	A12(2)AGHGQDIL<	72	A12(6)TALGAILK<
25	A 1(3)GHGQDILI<	73	A 1(7)ALGAILKK<
26	A 2(3)HGQDILIR<	74	A 2(7)LGAILKKK<
27	A 3(3)GQDILIRL<	75	A 3(7)GAILKKKG<
28	A 4(3)QDILIRLF<	76	A 4(7)AILKKKGH<
29	A 5(3)DILIRLFK<	77	A 5(7)ILKKKGHH<
30	A 6(3)ILIRLFKS<	78	A 6(7)LKKKGHHE<
31	A 7(3)LIRLFKSH<	79	A 7(7)KKKGHHEA<
32	A 8(3)IRLFKSHP<	80	A 8(7)KKGHHEAE<
33	A 9(3)RLFKSHPE<	81	A 9(7)KGHHEAEL<
34	A10(3)LFKSHPET<	82	A10(7)GHHEAELK<
35	A11(3)FKSHPETL<	83	A11(7)HHEAELKP<
36	A12(3)KSHPETLE<	84	A12(7)HEAELKPL<
37	A 1(4)SHPETLEK<	85	A 1(8)EAELKPLA<
38	A 2(4)HPETLEKF<	86	A 2(8)AELKPLAQ<
39	A 3(4)PETLEKFD<	87	A 3(8)ELKPLAQS<
40	A 4(4)ETLEKFDR<	88	A 4(8)LKPLAQSH<
41	A 5(4)TLEKFDRF<	89	A 5(8)KPLAQSHA<
42	A 6(4)LEKFDRFK<	90	A 6(8)PLAQSHAT<
43	A 7(4)EKFDRFKH<	91	A 7(8)LAQSHATK<
44	A 8(4)KFDRFKHL<	92	A 8(8)AQSHATKH<
45	A 9(4)FDRFKHLK<	93	A 9(8)QSHATKHK<
46	A10(4)DRFKHLKT<	94	A10(8)SHATKHKI<
47	A11(4)RFKHLKTE<	95	A11(8)HATKHKIP<
48	A12(4)FKHLKTEA<	96	A12(8)ATKHKIPI<

Figure 18.2.3 *(continued)*

Wash the pins

11. Remove the block of pins from the amino acid solutions and, if the next cycle is to start immediately, place the block in a methanol bath in which the pins are immersed to half their height (tips are fully immersed). Wash with agitation for 5 min. Flick off excess methanol and allow to air dry for 2 min.

12. Place the block in a DMF bath in which the pins are immersed to half their height (tips are fully immersed). Wash with agitation for 5 min.

    If extra pins are to be added as synthesis progresses (if the peptides being made differ in length), add the pins to the appropriate spaces (see Fig. 18.2.5).

13. Repeat steps 4 through 12 for each cycle of amino acid addition. Follow the synthesis schedule for preparing Fmoc–protected amino acid solutions and for depositing

GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1 Page 3
Bulk solutions for activator and/or additives (150 wells)
Chemistry Group 1 data for synthesis coupling 1
Activator : DIC requires 567.9 mg in 9.0 ml of DMF
Additive 1: HOBt requires 816.4 mg in 35.5 ml of DMF

WEIGHTS FOR INDIVIDUAL AMINO ACID SOLUTIONS

AA	#	Amino acid description	Batch	Weight (mg) Target	Weight (mg) Actual	DIC (ml)	HOBt (ml)
A	17	Fmoc-L-Ala-OH.H2O	.....	155.2	.....	0.94	3.77
D	6	Fmoc-L-Asp(OtBu)-OH	.....	75.1	.....	0.36	1.46
E	12	Fmoc-L-Glu(OtBu)-OH.H2O	.....	150.8	.....	0.68	2.72
F	6	Fmoc-L-Phe-OH	.....	70.7	.....	0.36	1.46
G	14	Fmoc-Gly-OH	.....	116.7	.....	0.79	2.30
H	12	Fmoc-L-His(Boc)-OH.5DCM	.....	176.8	.....	0.68	2.72
I	9	Fmoc-L-Ile-OH	.....	92.3	.....	0.52	2.09
K	19	Fmoc-L-Lys(Boc)-OH	.....	245.4	.....	1.05	4.19
L	17	Fmoc-L-Leu-OH	.....	166.5	.....	0.94	3.77
M	2	Fmoc-L-Met-OH	.....	28.8	.....	0.16	0.62
N	2	Fmoc-L-Asn(Trt)-OH	.....	46.2	.....	0.16	0.62
P	4	Fmoc-L-Pro-OH	.....	43.9	.....	0.26	1.04
Q	5	Fmoc-L-Gln(trt)-OH	.....	95.4	.....	0.31	1.25
R	4	Fmoc-L-Arg(PMC)-OH.3IPE	.....	90.4	.....	0.26	1.04
S	5	Fmoc-L-Ser(tBu)-OH	.....	59.9	.....	0.31	1.25
T	5	Fmoc-L-Thr(tBu)-OH	.....	62.1	.....	0.31	1.25
V	7	Fmoc-L-Val-OH	.....	70.8	.....	0.42	1.67
W	1	Fmoc-L-Trp(Boc)-OH	.....	27.0	.....	0.10	0.41
Y	3	Fmoc-L-Tyr(tBu)-OH	.....	47.7	.....	0.21	0.83

Figure 18.2.4 This page of the synthesis schedule is used for the preparation of activated amino acid solutions. It shows the amounts of each amino acid (represented by the single letter code, A through Y, along the left-hand margin), activator (diisopropylcarbodiimide [DIC] in dimethylformamide [DMF]), and catalyst (additive; 1-hydroxybenzotriazole [HOBt] in DMF) needed for the first amino acid coupling cycle. (In this example the amounts are calculated for a 250-µl reaction volume.) The amino acid powder is dissolved in the HOBt/DMF solution (right-hand column) before activation with DIC/DMF solution.

```
GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1        Page 4
        PIN POSITIONS for Synthesis coupling  1
                        NEW PIN POSITIONS
A 1(1) TO B 6(5)

              Well positions for amino acid dispensing

A  A10(1)  A 2(2)  A 5(2)  A12(4)  A 4(5)  A 6(6)  A 9(6)
     A 7(7)  A 1(8)  A 5(8)  B11(1)  B 2(3)  B 4(3)  B 7(3)
     B11(3)  B 8(4) TO B 9(4)

D  A 3(2)  A10(2)  A 3(4)  A 7(5)  B 3(3)  B 6(4)

E  A 1(2)  A 9(3)  A12(3)  A11(4)  A 1(5)  A 6(5)  A 6(7)
     A 8(7)  B 6(1)  B10(1)  B 1(4)  B 1(5)

F  A 4(3)  A 2(4)  A 5(4)  B 7(1)  B12(2)  B 3(4)

G  A 1(1) TO A 2(1) A 6(2)  A 8(2)  A12(5)  A 8(6)  A 3(7)
     B 1(1) TO B 2(1) B10(2)  B 1(3)  B 6(3)  B 3(5)  B 6(5)

H  A 7(1)  A 7(2)  A 7(3)  A 7(4)  A11(5)  A 4(7) TO A 5(7)
     A 4(8)  A 8(8)  B 2(2)  B 5(2)  B 8(2)

I  A11(2)  A 1(3)  A10(6)  A10(8)  A12(8)  B 8(1)  B12(1)
    TO B 1(2) B 7(4)

K  A11(1)  A 5(3)  A 1(4)  A 6(4)  A 9(4)  A 3(5)  A 9(5)
    TO A10(5) A12(6) TO A 2(7) A10(7)  A 7(8)  A 9(8)  B 3(1)
     B10(3)  B 5(4)  B10(4)  B12(4)

L  A 4(1)  A 6(1)  A12(2)  A 3(3)  A11(3)  A 8(4)  A 8(5)
     A 4(6)  A 7(6)  A11(6)  A 9(7)  A12(7)  B 5(1)  B 4(2)
     B12(3)  B 2(4)  B 2(5)

M  A 2(5)  B 8(3)

N  B11(2)  B 9(3)

P  A 8(3)  A11(7)  A11(8)  B 9(2)

Q  A 3(1)  A 9(2)  A 2(8)  B 5(3)  B 5(5)

R  A 2(3)  A 4(4)  B 7(2)  B 4(4)

S  A 6(3)  A 5(5)  A 3(8)  B 9(1)  B 6(2)

T  A10(3)  A10(4)  A 2(6)  A 5(6)  A 6(8)

V  A 5(1)  A 8(1)  A12(1)  A 4(2)  A 1(6)  A 3(6)  B 3(2)

W  A 9(1)

Y  B 4(1)  B11(4)  B 4(5)
```

Figure 18.2.5 This page of the synthesis schedule identifies which wells of the reaction tray receive which activated amino acid solution. Each amino acid solution is identified along the left-hand margin using the single-letter amino acid code. Individual reaction trays and pin holders (A or B in this case) are identified by letters of the alphabet, and the paired numbers—e.g., 10(1) for well 10H—identify individual wells within reaction trays according to the numbering system illustrated in Figure 18.2.6.

aliquots of the activated amino acids to the appropriate wells of the reaction tray for each cycle.

Two coupling cycles can be carried out during a normal working day, and a third coupling can be carried out overnight, so a total of three amino acids can be added to each pin during a 24-hr period.

14. Deprotect the final Fmoc amino acid by repeating steps 4 through 8. Then proceed with step 15 or step 16.

15. *Optional:* For B cell epitope scanning or for T helper cell epitope scanning, the N-terminus of the peptide can be capped by acetylation (see Support Protocol 2). To allow later recapture onto avidin, the N-terminus of the peptide can be capped with biotin or long-chain biotin (see Support Protocol 3).

Deprotect the side chains

16. Dispense the required volume of SCD solution into a bath or tubes (1.5 ml/tube) and fully immerse the peptide-bearing portion of the pins. Cover bath or cap tubes and incubate 2.5 hr at 20° to 25°C.

CAUTION: SCD solution is a toxic, corrosive liquid.

For blocks where the peptide is to remain attached to the pin during side chain deprotection (i.e., NCP, GAP, and DKP kits), side chain deprotection is carried out in a bath of reagent. For pins where the peptide is simultaneously side chain deprotected and cleaved from the pin (MPS kits), the process is carried out in individual 10-ml capped conical polypropylene centrifuge tubes that become the containers for the recovered peptide.

17a. *For NCP, GAP, or DKP kits:* Wash the pins 3 times in acidified methanol to remove the SCD solution prior to further treatment.

17b. *For MPS kit:* In a good chemical fume hood, reduce the volume of SCD solution containing cleaved peptide to ~0.1 ml either with a gentle stream of dry nitrogen gas or in a centrifugal vacuum drier (e.g., Speedvac) equipped to handle corrosive fumes. Precipitate the peptide in the remaining solution with 8 ml of 1:2:0.003 ether/petroleum ether/2-ME. Decant and discard the supernatant, and wash the

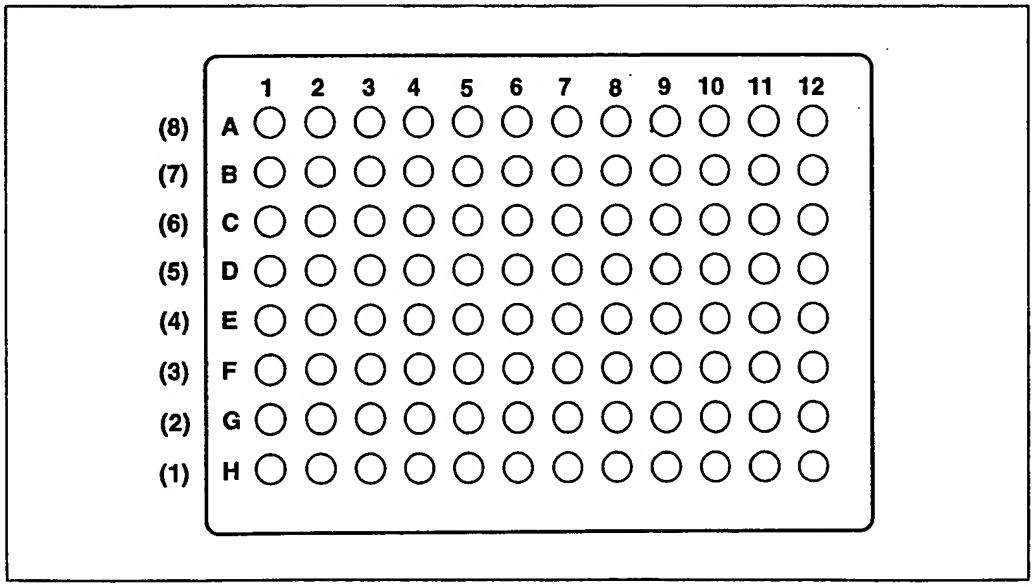

Figure 18.2.6 The numbering system for pins and reaction trays for Chiron Technologies' multipin synthesis system. Each well is identified by a pair of numbers rather than a number and a letter, e.g, 1(1). The first number identifies the column number; the second (in parentheses) identifies the lettered row, beginning with H as (1) and ending with A as (8).

precipitated peptide with 4 ml of 1:2 ether/petroleum ether. Dry the precipitate with a gentle stream of dry nitrogen.

CAUTION: Ether/petroleum ether/2-ME and ether/petroleum ether solutions are highly flammable.

These dry peptides can now be redissolved for assay purposes or may be further purified, e.g., by HPLC (see UNIT 11.6).

Prepare the peptides

18a. *For GAP kits:* Add 0.7 ml of 0.1 M NaOH to each tube of a rack of 96 1-ml polypropylene tubes. Place the pins into the solution and incubate ~1.5 hr to cleave the peptides from the pins. Immediately after cleavage, neutralize with one equivalent of 0.1 M acetic acid.

Alternatively, the 0.1 M NaOH solution can contain 40% (v/v) acetonitrile to facilitate solubilization of the more hydrophobic peptides.

The incubation time can be shorter if the tubes are sonicated during cleavage.

18b. *For DKP kits:* Cleave the peptide from the pin in a suitable, reasonably well-buffered solution with a pH >7 overnight (16 hr).

The solution for cleavage—e.g., 0.1 M sodium phosphate, pH 8, or 0.1 M HEPES, pH 8—can be chosen to be compatible with the assay for which the peptides will be used; the solution should have a buffering capacity of ~0.05 M.

Again, an organic modifier such as acetonitrile can be added to the cleavage solution to facilitate solubilization of the more hydrophobic peptides.

18c. *For NCP kits:* Prepare the pins for binding assays by floating the block, pin-side down, in sonication buffer and sonicating 10 min at ~60°C. Rinse the pins first in water, then in 20° to 45°C methanol for immediate use in an assay or air dry the pins for storage until they are used in an assay.

PREPARING ACTIVATED Fmoc–PROTECTED AMINO ACID SOLUTIONS

SUPPORT PROTOCOL 1

Activated 9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acid solutions are prepared in two steps. First, the protected amino acid is dissolved in a solution of the catalyst, 1-hydroxybenzotriazole (HOBt) in dimethylformamide (DMF). Then, just before dispensing, the amino acid is activated by adding an activating agent. The following procedure illustrates the use of diisopropylcarbodiimide (DIC) as the activating reagent. DIC is a liquid and can be measured by volume.

***Additional Materials** (also see Basic Protocol)*

9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acids with side-chain-protecting groups (Sigma, Bachem, Novachem, or Chiron Technologies), stored at 4°C
Catalyst: 1-hydroxybenzotriazole (HOBt)
Activating agent: diisopropylcarbodiimide (DIC)
Dimethylformamide (DMF), amine-free
Ethanol, analytical reagent grade
5- or 10-ml glass, polyethylene, or polypropylene bottles with inert (e.g., polythylene, Teflon) lids and liners

1. Remove the Fmoc–protected amino acids from the refrigerator and allow them to come to room temperature before weighing them out.

Warming the containers to room temperature avoids the possibility of uptake of moisture from the air onto the cold solids.

The side chains of the amino acids must also be protected during peptide synthesis: t-butyl ether is used for serine, threonine, and tyrosine; t-butyl ester is used for aspartic acid and glutamic acid; t-butoxycarbonyl (t-Boc) is used for lysine, histidine, and tryptophan; 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) is used for arginine; and trityl (Trt) is used for cysteine. If benzotriazolyl-N-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) or benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) activation is to be used, trityl protection should be used for asparagine and glutamine. BOP and PyBOP require greater care in handling and different protection should be used for some amino acids.

2. Weigh individual amino acids and HOBt using the quantities specified for the current synthesis cycle (e.g., see Fig. 18.2.4) into separate appropriately sized clean and dry glass, polyethylene, or polypropylene bottles. Rinse the spatula with ethanol and dry it after weighing each reagent.

   The bottle caps and inserts must be inert to any of the reagents or solvents used in making up the activated solutions (they must be Teflon, not rubber).

   Care should be taken to avoid cross-contamination of amino acids by rinsing the spatula in ethanol between weighings (the spatula must be dry before each use) and by making sure that all lids, as well as the containers, are labeled so they can be replaced on the correct bottles after weighing has been completed.

3. Measure the appropriate amount of DIC (see "Activator" as in Fig. 18.2.4).

   DIC is a liquid so it is more convenient to measure it by volume rather than by weight. Multiply the indicated weight by 1.23 (based on the density of DIC, 0.815 g/ml) as a conversion factor from the calculated weight to get the required volume of DIC in microliters.

4. Prepare HOBt and DIC solutions, by pipetting the appropriate volumes of purified (amine-free) DMF as shown on the synthesis schedule (e.g., see Fig. 18.2.4).

   Both reagents should be fully dissolved in the DMF before using them to prepare the activated amino acid solutions.

5. Add the specified volume of HOBt/DMF solution (e.g., Fig. 18.2.4, column headed "HOBt") to the individual amino acids.

   Make sure the amino acids are completely dissolved before adding the activator solution.

   Unactivated amino acid solutions may be stored a few days at 4°C.

6. Activate the individual amino acid solutions by adding the specified volume of DIC/DMF solution to each amino acid solution (e.g., Fig. 18.2.4, column headed "DIC"). Mix thoroughly and use immediately for peptide synthesis.

   Activated amino acids should be prepared immediately before use and any excess should be discarded.

SUPPORT PROTOCOL 2

N-TERMINAL ACETYLATION OF PEPTIDES

N-terminal capping of the peptides is carried out after a final 9-fluorenylmethyloxycarbonyl (Fmoc)–deprotection cycle and prior to side chain deprotection. The process is similar to coupling of amino acids, except that in the case of acetylation the active reagent can be acetic anhydride rather than acetic acid. Acetic anhydride does not require activation. If acetic anhydride is not available, simply use acetic acid as if it were an amino acid (see Support Protocol 3).

Additional Materials (also see Basic Protocol)

Acetylation solution (see recipe), prepared just before use
Pins with completed peptides (see Basic Protocol)

1. Add freshly prepared acetylation solution to appropriate bath container.
2. Immerse the pins with completed peptides in acetylation solution and incubate 90 min at room temperature.
3. Wash the pins in a methanol bath and air dry.

The pins can now be used for side chain deprotection (Basic Protocol, step 16).

N-TERMINAL BIOTINYLATION OF PEPTIDES

SUPPORT PROTOCOL 3

Biotin can also be coupled to the N-terminus of peptides after N-terminal deprotection and before side chain deprotection. The reagent is used as if it were an amino acid, using the same solvent, activating agent, and catalyst.

***Additional Materials** (also see Basic Protocol)*

Biotin or long-chain biotin
Dimethylformamide (DMF), amine-free
Diisopropylcarbodiimide (DIC)
Pins with completed peptides (see Basic Protocol)

1. Dissolve biotin in amine-free DMF to a concentration of 125 mM.
2. Prepare a 10× solution of DIC (activating agent) by dissolving 158 mg DIC in 1 ml DMF and prepare a 10× solution of HOBt (catalyst) by dissolving 192 mg HOBt in 1 ml DMF.
3. Activate the biotin with the 10× concentrate solutions of activation agent and catalyst (80:10:10 [v/v/v]).
4. Dispense 150 µl/well (for gears) or 450 µl/well (for macrocrowns) into reaction trays.
5. Immerse the pins with completed peptides in the reaction tray and incubate ≥2 hr.
6. Wash the pins in methanol.

The pins can now be used for side chain deprotection (see Basic Protocol, step 16).

REAGENTS AND SOLUTIONS

*Use Milli-Q water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2E**; for suppliers, see **SUPPLIERS APPENDIX**.*

Acetylation solution

For 200 ml:
193 ml dimethylformamide (DMF)
6 ml acetic anhydride
1 ml *N*-ethyldiisopropylamine
Prepare immediately before use and discard after use

DO NOT expose pins to acetic anhydride at any other time except during acetylation. Also, do not store acetic anhydride anywhere near where peptide synthesis is performed.

The DMF does not need to be amine-free.

20% piperidine/DMF

Prepare a 20% (v/v) solution of the best quality piperidine available in analytical reagent–grade dimethylformamide (DMF). Prepare a fresh solution for each synthesis (solution can be reused several times within a synthesis). Store at room temperature in an amber bottle containing activated molecular sieves to remove moisture.

CAUTION: This solution is highly flammable and toxic.

continued

If high-quality piperidine is not available, it may have to be treated with solid sodium hydroxide and redistilled.

DMF need not be amine-free.

Side chain deprotecting (SCD) solution

33 parts (v/v) trifluoroacetic acid
1 part (v/v) ethanedithiol
2 parts (v/v) anisole
2 parts (v/v) thioanisole
2 parts (v/v) H_2O
Prepare immediately before use and do not store or reuse

CAUTION: This solution is corrosive and extremely malodorous. Contamination of the laboratory, especially with ethanedithiol, should be avoided. Wipe the outside of ethanedithiol-contaminated equipment or containers with dilute, 0.1% aqueous hydrogen peroxide to oxidize ethanedithiol to a nonodorous compound before removing the container from the fume hood. DO NOT allow hydrogen peroxide to contact other readily oxidizable materials or reagents.

Sonication buffer

1% (w/v) SDS
0.1 M sodium phosphate buffer, pH 7.2 (*APPENDIX 2E*)
0.1% (v/v) 2-mercaptoethanol (2-ME)
Store at room temperature up to 1 week

CAUTION: Before discarding sonication buffer, destroy remaining 2-ME by adding 2 ml 30% hydrogen peroxide per liter of buffer.

COMMENTARY

Background Information

The multipin method was developed by Dr. H.M. Geysen and coworkers (Geysen et al., 1984, 1987) as a scanning method for linear antibody-defined epitopes. Eventually in the late 1980s, the method was adapted to parallel synthesis of cleaved (soluble) peptides (Maeji et al., 1990), opening the way for systematic scanning of T helper (Reece et al., 1993) and cytotoxic epitopes (Burrows et al., 1994). Initially only suitable for synthesis of short peptides (up to 10 amino acid residues), the method can now routinely produce peptides of up to 20 residues of acceptable quality for initial screening experiments (Valerio et al., 1993).

Critical Parameters

Successful peptide synthesis requires reagents of a quality appropriate to the particular step, and the careful application of those reagents. For example, the protected amino acids need to be free of reactive counterions such as dicyclohexylamine (DCHA), contaminating unprotected amino acid, isomers such as the D-amino acid, and water. Check carefully that the amino acid as supplied is EXACTLY the same as specified in the manual or on the software. Apart from quality testing each amino acid, the best assurance of quality is to buy only from reputable suppliers.

Dimethylformamide (DMF) is the primary solvent for carrying out reactions (couplings) on pins. Its low volatility and moderate polarity make it suitable for dissolving the amino acids and solvating the graft polymer/growing peptide on the pin surface. Purity is not critical for some (washing) steps, but is critical for the DMF used just before and during amino acid coupling. Presence of excessive amine in the DMF results in loss of activated amino acid because the amino acid couples to the amine rather than to the peptide on the pin. Fortunately, the pin system allows use of substantial molar excesses of incoming amino acid (typically 6- to 1000-fold), so loss of some amino acid is not disastrous. Fresh DMF of the best available grade should be used for the coupling, and it is recommended that the amine level be tested using the FDNB test (Stewart and Young, 1984).

Liberal use is made of methanol as a washing solvent. Analytical reagent grade methanol is readily available at low cost in large containers (20 or 200 liters) and is relatively easy to dispose of. It is possible to reduce the use of methanol by reusing it for washes: the last wash

bath in any series should be in fresh (pure) methanol. In the next round of washes, the former last bath is then reassigned as the second-to-last wash, the previously second-to-last bath becomes the third-to-last, and so on. For each synthesis cycle, the first wash bath in the series is the one which is discarded. The presence of methanol is undesirable during reactions on the pins, but as it evaporates readily it can be easily removed by standing the block in a moving stream of air, such as the opening of an operating chemical fume hood. Methanol will dry more rapidly and the methanol-washed pins will take up less moisture from the air if the methanol is warm (e.g., prewarmed to 45°C in a closed bottle in a water bath).

Other solvents (e.g., ether, petroleum ether, acetonitrile) should be the best available grade.

Carrying out the correct synthesis of the peptides requires that all steps are performed with a very high level of attention to detail. All cyclically repeated steps (washes and deprotections) must be performed, and the activation and dispensing of the amino acids for each coupling cycle must be carried out exactly, or the peptides made may have the incorrect sequence, may be missing an amino acid, or may be truncated. Computerized equipment is available for assisting with the accurate dispensing of amino acids to the wells in a reaction tray (e.g., "Pin-Aid," Chiron Technologies; Carter et al., 1992). The growing peptides must not be subjected to conditions that would prematurely block or deprotect the side chains (for example, from premature exposure to acetic anhydride or trifluoroacetic acid, which should be stored well away from where peptide synthesis is being performed).

As a spot test for correct completion of all the steps of synthesis, it is wise to synthesize controls on each block of 96 pins. For noncleavable peptides, these controls can be peptide sequences that can be probed with an antibody known to react with the peptide. In this case, one of the two peptides should be a negative control, such as a randomized sequence. For cleavable peptides, the quantity and quality of the controls can be monitored by the usual techniques of HPLC (*UNIT 11.6*), amino acid analysis (*UNIT 11.9*), and mass spectrometry (Chapter 16). Ultimately, proof that an assay result is a function of the particular peptide made has to rely on a confirmatory experiment carried out with more highly-characterized peptide or on analysis of a sample of the particular peptide used in the experiment.

Once peptides have been made, they need to be handled and stored carefully to prevent degradation. Noncleavable peptides (pins) should be stored dry in a refrigerator after removal of any bound protein. If stored with desiccant they should be stable for months to years. Cleaved peptides can be stored frozen or as dry powder. After a long period of storage, it is wise to reassay controls or confirm the quality of the stored peptide by analysis.

Another parameter critical to data from large numbers of peptides is to ensure that the identity of each peptide is properly tracked and that activity is not ascribed to the wrong peptide. Consistent use of the 8 × 12 microtiter plate format for synthesis, storage, assay, and use of computerized records for tracking all three processes can help avoid mistakes. Tracking and control is particularly easy if the assay data is read directly from a microtiter plate reader to a computer that is programmed with the peptide information because this method avoids manual data transcription.

Anticipated Results

For a noncleavable pin-peptide synthesis, two control peptides, one of which is reactive with a monoclonal antibody in ELISA and the other serving as a nonbinding peptide control, should show the specific binding expected based on past data. For cleaved peptides, the yield of control peptide should be in the range expected from the stated pin loading (substitution level), e.g., 1 μmol for GAP and DKP kits or 5 μmol for the MPS kit. Purity of the cleaved controls should be consistent with the results of previous batches and should be of an acceptable standard.

Testing of a systematic set of peptides in a bioassay can give data that is interpretable without recourse to additional controls, because a systematic set of peptides through a protein includes many sequences that are unlikely to be reactive sequences, i.e., they act as internal negative controls. Figure 18.2.7 shows one set of ELISA data from scanning noncleaved peptides with a monoclonal antibody. In screening for T helper cell responsiveness it is critical to include many control cultures, not only controls with no peptide added but also controls with nonstimulatory peptide. Systematic sets of peptides automatically include such controls (Reece et al., 1994).

Time Considerations

If amino acid coupling is carried out at 3 cycles/day, which can fit into a conventional working day, then it will take up to 2 weeks to make a set of 15-mers, as there is extra time required for side chain deprotection and drying down (depending on the peptide format). Although this may seem slow, the fact that hundreds or thousands of peptides can be made simultaneously means that a project requiring large numbers of peptides is completed in a very short time. Indeed, the rate-limiting step may be the time it takes to carry out the assays on the large number of peptides when they become available.

From this perspective, biotinylated peptides produced on glycine acid peptide (GAP), diketopiperazine (DKP), or multiple peptide syn-

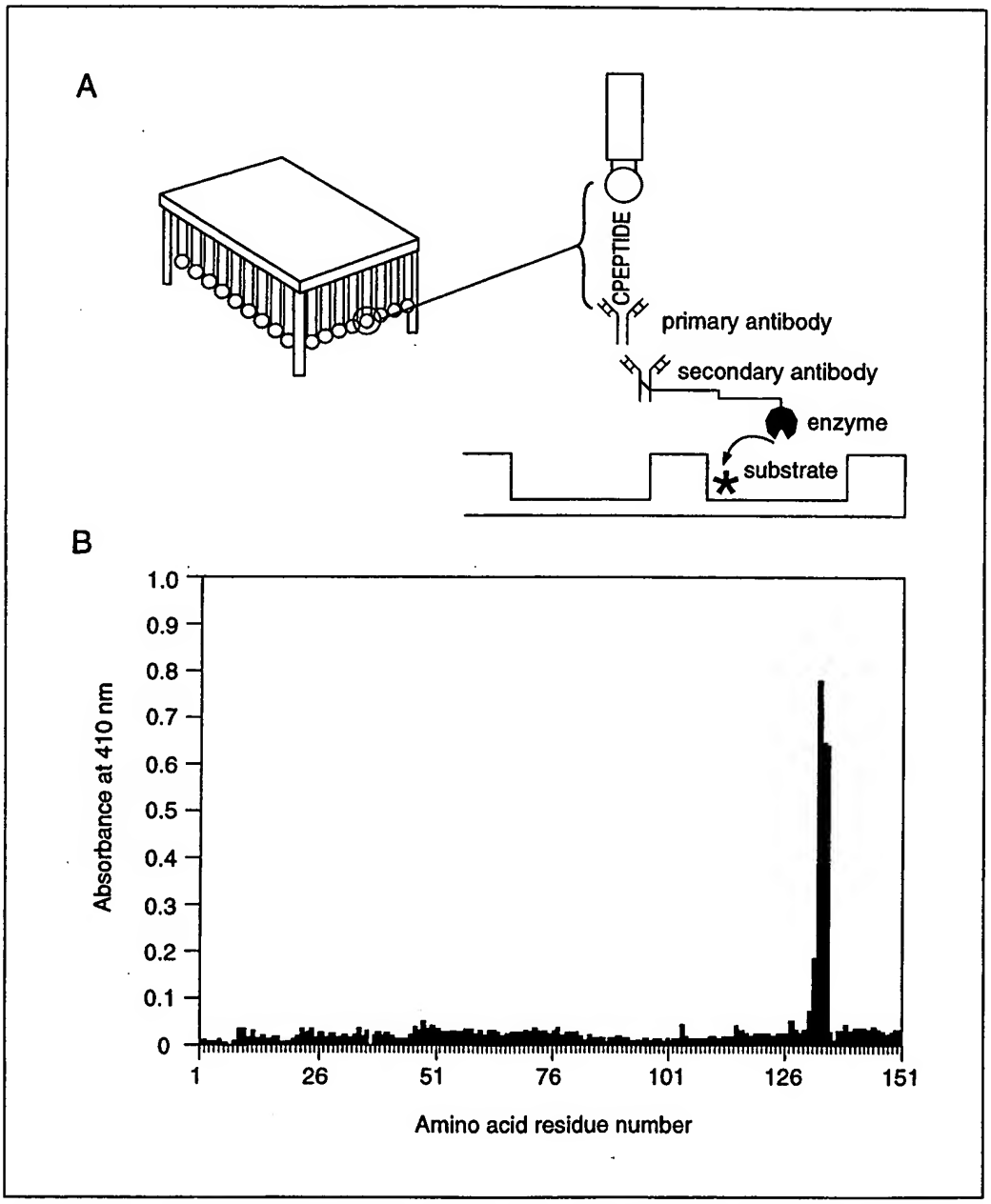

Figure 18.2.7 Multipin capture ELISA. (**A**) Setup for multipin capture ELISA. Pins (gears) with peptides covalently attached are incubated in primary antibody, secondary antibody, and substrate developer in ELISA plates. The absorbance is measured and the resulting absorbance values are graphed versus peptide number, corresponding to the N-terminal residue number of the peptide in the protein sequence. (**B**) Peptide pin capture ELISA results with a monoclonal antibody against pins bearing octamer peptides of gonococcal pilin protein. All the peptides that show high readings contain a significant portion of the epitope. (Diagram courtesy of Dr. Fred Cassels, Walter Reed Army Institute of Research, Washington, D.C.)

thesis (MPS) pins have a great advantage over the noncleavable peptide (NCP) pin-bound peptides, as the latter can only be assayed once a day, whereas hundreds of parallel assays can be carried out on all biotinylated peptides at once. Reading data directly into a computer enables the massive amounts of data to be stored efficiently for later analysis.

Dispensing amino acids can be carried out efficiently by two people, one reading out the position into which the amino acid is to be dispensed and the other doing the actual dispensing. The passive partner (reader) can also act as a cross-checker to ensure no mistakes are made. If a computer-controlled pointing device is used, accuracy is improved and dispensing becomes a one-person operation. For large syntheses (>200 peptides), it is important that the dispensing be fast and accurate so that three couplings can be carried out per day.

Literature Cited

Burrows, S.R., Gardner, J., Khanna, R., Steward, T., Moss, D.J., Rodda, S., and Suhrbier, A. 1994. Five new cytotoxic T cell epitopes identified within Epstein-Barr virus nuclear antigen 3. *J. Gen. Virol.* 75:2489-2493.

Carter, J.M., VanAlbert, S., Lee, J., Lyons, J., and Deal, C. 1992. Shedding light on peptide synthesis. *Bio/Technology* 10:509-513.

Fauchere, J.L. and Pliska, V. 1983. Hydrophobic parameters of amino acid side chains from the partitioning of *N*-acetyl-amino-acid amides. *Eur. J. Med. Chem.* 18:369-375.

Geysen, H.M., Meloen, R.H., and Barteling, S.J. 1984. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proc. Natl. Acad. Sci. U.S.A.* 81:3998-4002.

Geysen, H.M., Rodda, S.J., Mason, T.J., Tribbick, G., and Schoofs, P.G. 1987. Strategies for epitope analysis using peptide synthesis. *J. Immunol. Methods* 102:259-274.

Maeji, N.J., Bray, A.M., and Geysen, H.M. 1990. Multi-pin peptide synthesis strategy for T cell determinant analysis. *J. Immunol. Methods* 134:23-33.

Mutch, D.A., Rodda, S.J., Benstead, M., Valerio, R.M., and Geysen, H.M. 1991. Effects of end groups on the stimulatory capacity of minimal length T cell determinant peptides. *Pept. Res.* 4:132-137.

Reece, J.C., Geysen, H.M., and Rodda, S.J. 1993. Mapping the major human T helper epitopes of tetanus toxin: The emerging picture. *J. Immunol.* 151:6175-6184.

Reece, J.C., McGregor, D.L., Geysen, H.M., and Rodda, S.J. 1994. Scanning for T helper epitopes with human PBMC using pools of short synthetic peptides. *J. Immunol. Methods* 172:241-254.

Rink, H. 1987. Solid-phase synthesis of protected peptide fragments using a trialkoxydiphenylmethylester resin. *Tetrahedron Lett.* 28:3787-3790.

Stewart, J.M. and Young, J.D. 1984. Solid Phase Peptide Synthesis, 2nd ed. Pierce Chemical Co., Rockford, Ill.

Valerio, R.M., Bray, A.M., Campbell, R.A., Dipasquale, A., Margellis, C., Rodda, S.J., Geysen, H.M., and Maeji, N.J. 1993. Multipin peptide synthesis at the micromole scale using 2-hydroxyethyl methacrylate grafted polyethylene supports. *Int. J. Pept. Protein Res.* 42:1-9.

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(54) Title: INHIBITORS OF SEPARIN, METHOD FOR IDENTIFYING THEM AND USES

(57) Abstract

Method for identifying compounds that interfere with or modulate sister chromatid separation in animal or plant cells by modulating a protease with separin–like cysteine endopeptidase activity. Inhibitors of separin activity are useful in cancer therapy, to prevent birth defects and to increase ploidy in plants.

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Title: INHIBITORS OF SEPARIN, METHOD FOR IDENTIFYING THEM AND USES

The invention relates to compounds influencing mitosis and meiosis in eukaryotic cells and methods for identifying such compounds. In particular, the invention largely relates to the treatment and prevention of human conditions by modulating sister chromatid segregation.

During the process of cell division, sister chromatids are pulled to opposite halves of the cell by microtubules emanating from spindle poles at opposite sides of the cell. One set of microtubules inter-digitates with others emanating from the opposite pole. Their role is to keep (and drive) the two poles apart. Meanwhile, a second set of microtubules attaches to chromosomes via specialized structures called kinetochores and pulls them towards the poles. Sister chromatids segregate away from each other because their kinetochores attach to microtubules emanating from opposite poles (Rieder et al., 1998). Chromosomes are not mere passengers during this process. During metaphase, the tendency of microtubules to move sisters apart is counteracted by cohesion holding sisters together. Cohesion therefore generates the tension by which cells align sister chromatids on the metaphase plate. Were sisters to separate before spindle formation, it is difficult to imagine how cells could distinguish sisters from chromatids that were merely homologous. The sudden loss of cohesion, rather than an increase in the exertion of microtubules, is thought to trigger sister separation during anaphase (Miyazaki et al., 1994). Cohesion also prevents chromosomes falling apart due to double strand breaks and facilitates their repair using recombination.

To avoid missegregation of chromosomes, anaphase must only be initiated after sister chromatids of each duplicated chromosome have attached to opposite poles of the mitotic spindle. Microtubules are thought to "find" kinetochores by a "search and capture" mechanism which cannot be completed simultaneously for all chromosomes (Hayden et al., 1990; Merdes and De Mey, 1990). Cells therefore possess regulatory mechanisms that delay sister chromatid separation until the last chromosome has achieved bipolar attachment. The dissolution of sister chromatid cohesion at the metaphase to anaphase transition is therefore a highly regulated step during the eukaryotic cell cycle.

Sister chromatid cohesion depends on a multi-subunit complex called cohesin (Losoda et al., 1998), which contains at least four subunits: Smc1p, Smc3p, Scc1p, and Scc3p, all of which are conserved between yeast and humans. It is likely, but not yet proven, that cohesin is a key constituent of the tether that holds sister chromatids together. The association between cohesin and chromosomes has recently been shown to depend on the Scc2 (Mis4) protein. Cohesion is established during DNA replication (Uhlmann and Nasmyth, 1998). It has been recently shown that the Eco1 (Ctf7) protein is required for the establishment of cohesion during S phase but unlike cohesin is not required to maintain cohesion during G2 and M phases. In yeast, cohesin remains tightly associated with chromosomes until metaphase; that is, it is present on chromosomes during their alignment during metaphase. In animal cells, however, the bulk of cohesin dissociates from chromosomes during prophase (Losada et al., 1998). It is unclear how much cohesin, if any, remains on chromosomes during metaphase. The nature of the link that holds sister chromatids together during metaphase in animal cells is therefore unclear. It could either involve a small fraction of cohesin that remains associated with chromosomes or some other protein complex.

In yeast, at least two of cohesin's subunits, Scc1p and Scc3p, suddenly disappear from chromosomes at precisely the point at which sister chromatids separate (Michaelis et al., 1997). This has led to the notion that a sudden change in the state of cohesin might trigger the onset of anaphase, at least in yeast. In Drosophila, the meiS332 protein, which binds to chromosomes during prometaphase, also disappears at the onset of anaphase. MeiS332 is required for sister chromatid cohesion during meiosis but not during mitosis (Moore et al., 1998; Kerrebrock et al., 1995). MeiS332 is probably not a cohesin subunit nor is it apparent whether homologous proteins exist in humans.

Both the dissociation of Scc1p from chromosomes and the separation of sister chromatids are dependent on a specialized sister separating protein (a separin) called Esp1p (Ciosk et al., 1998). Separins homologous to Esp1 exist in the fission yeast Schizosaccharomyces pombe, in the fungus Aspergillus nidulans, in the nematode worm Caenorhabditis elegans, the fruit fly Drosophila melanogaster, in the frog Xenopus laevis, in the plant

Arabidopsis thaliana, and in man. This strongly suggests that separins have a fundamental role in chromosome segregation that is conserved between plants, fungi, and animals. Esp1 is tightly bound by an inhibitory protein called Pds1p whose destruction shortly before the metaphase to anaphase transition is triggered by ubiquitination mediated by the anaphase promoting complex (APC) (Cohen-Fix et al., 1996). The APC is large multi-subunit complex, most of whose subunits are conserved between yeast and humans. Together with activator proteins called Cdc20p and Cdh1p, it mediates the ubiquitination and thereby destruction of many different cell cycle proteins, including anaphase inhibitors like Pds1 and mitotic cyclins. Pds1 destruction is mediated by a form of the APC bound by the activator Cdc20. This form is called APC^{Cdc20}. (For a review, see Peters, 1998)

Proteins with similar properties to Pds1 have been found in fission yeast (Cut2p), in Xenopus, and in humans (Funabiki et al., 1996; Zou et al., 1999). The APC is essential for sister chromatid separation in most if not all eukaryotic organisms. In yeast, it is clear that its main role in promoting sister separation is to destroy Pds1, which liberates Esp1 and allows it to destroy sister chromatid cohesion, possibly by destroying the physical links between sisters mediated by cohesin.

It was an object of the invention to further eluciate the mechanism of sister chromatid separation.

In particular, it was an object of the invention to understand the mechanism by which Esp1p mediates the dissocation of Scc1p from chromosomes in the budding yeast in order to take advantage of this mechanism by using it as a target in human therapy, in particular of cancer therapy, and as a target in all other situations where modulation of sister chromatid separation is therapeutically or otherwise beneficial.

To solve the problem underlying the present invention, the following approach was taken:

The observation that the dissociation of Scc1p from chromosomes at the onset of anaphase is dependent on Esp1 suggested that Esp1 might either have a direct role in this process or that Esp1 might be indirectly involved

by initiating a process that leads to Scc1p's dissociation. It was found in experiments of the present invention that Esp1 also prevents association of Scc1p with chromosomes during G1 (see Example 1), which strongly suggests that Esp1's role might be very direct. Scc1p is an unstable protein which is rapidly destroyed following its dissociation from chromosomes at the onset of anaphase and which must be re-synthesised during late G1 during the next cell cycle in order for cohesion to be established at the next round of DNA replication (Michaelis et al., 1997) It was found that Scc1p synthesised during G2 is also capable of binding to yeast chromosomes but that it fails to produce cohesion under these circumstances (Uhlmann and Nasmyth, 1998). However, it was noted that Scc1p synthesised during early G1 binds to chromosomes poorly, if at all. As shown in Figure 1, inactivation of Esp1 permits the efficient association between Scc1p and chromosomes during early G1. The implication is that Esp1 not only triggers Scc1p's dissocation from chromosomes at the onset of anaphase but also prevents Scc1p's stable association with chromosomes during the subsequent G1 period. This strongly suggests that Esp1 has a fairly direct role in controlling the association between Scc1p and chromosomes.

Starting from this finding, an assay was established by which Esp1 activity could be measured in vitro. A crude preparation of yeast chromatin isolated from cells arrested in a metaphase-like state by nocodazole, was incubated with a soluble extract prepared from cells over-producing Esp1 from the GAL promoter (Figure 2). To detect Scc1p, cells were used whose Scc1 protein was tagged at its C-terminus with multiple HA or Myc epitopes, which can readily be detected with monoclonal antibodies. About 70% of the total Scc1p in nocodazole blocked cells is tightly associated with chromatin and is therefore present in the chromatin fraction that was used. Most of the Scc1p remains tightly associated with chromatin following incubation with an extract prepared from esp1-1 mutant cells but most disappears from the chromatin fraction upon incubation with extracts containing high levels of wild type Esp1 protein. Somewhat surprisingly, the Scc1p protein induced to dissociate from chromatin by Esp1 appeared in the "soluble" supernatant fraction as a cleaved product. The C-terminal fragments of this cleavage were detected by using as a substrate a C-terminally tagged Scc1 protein and N-terminal fragments using as substrate an N-terminally tagged Scc1 protein. The sizes of these cleavage products

suggested that Esp1 induces one or more specific cleavages of Scc1p within a 10 kd interval. This Esp1-dependent cleavage was inhibited by the addition of reticulate lysate that had translated Pds1 but not by an otherwise identical lysate that had translated an unrelated control protein. The Esp1 activity detected by the cleavage assay is therefore inhibited by Pds1, which demonstrates directly, for the first time, that Pds1 is indeed an inhibitor of Esp1p.

To address whether Esp1 induced cleavage of Scc1p also occurs in vivo at the onset of anaphase, a yeast strain was constructed in which expression of the APC activator Cdc20p is under control of the galactose inducible *GAL1-10* promoter. The strain also expressed an Scc1 protein tagged at its C-terminus with multiple HA or myc epitopes. Cells from this strain were arrested in metaphase by incubation in galactose free medium and then induced to embark on anaphase highly synchronously by the addition of galactose. Figure 3 shows that sister chromatids separate in most cells within 15 minutes and that Scc1p dissociates from chromosomes with similar if not identical kinetics. A low level of an Scc1 cleavage product was detected that is identical to that seen in vitro in cycling cells but none in cells arrested in metaphase. The cleavage product suddenly appeared upon induction with galactose with kinetics that were similar if not identical to the separation of sister chromatids and dissocation of Scc1p from chromosomes. To establish whether this in vivo cleavage was dependent on Esp1 activity the extent of Scc1p cleavage in wild type and esp1-1 mutants when released from cdc20 arrest at 35.5°C was compared (the restrictive temperature for esp1-1). The extent of Scc1p cleavage was greatly reduced in the esp1-1 mutant. It was concluded that Esp1 promotes the cleavage of Scc1p and its dissociation from chromosomes both in vivo and in vitro.

To address whether cleavage of Scc1 mediated by Esp1 is important either for sister chromatid separation or for Scc1p's dissociation from chromosomes, the cleavage site was mapped in order that it could then be mutated. An epitope tagged Scc1p protein from cells that had been stimulated to undergo anaphase by induction of Cdc20 expression was immunoprecipiated and the immunoprecipitated proteins were separated on SDS page. A short stretch of N-terminal amino acid sequence from the

C-terminal cleavage fragment was then determined by Edman degradation. This showed that cleavage in vivo had occured between a pair of arginines at positions 268 and 269. The N-terminal of these arginine residues was then mutated to aspartic acid and an HA tagged version then was expressed from the *GAL1-10* promoter in yeast cells whose endogenous Scc1 protein was myc tagged. Galactose induced expression of this single mutant protein had little effect on cell proliferation. To establish whether the mutation had indeed abolished cleavage, chromatin from cells expressing the mutant protein was isolated and used as a substrate in the Esp1 assay. This showed that cleavage at site 268 was indeed eliminated by the aspartic acid mutation. However, the mutated protein was still cleaved in an Esp1-dependent manner. The C-terminal cleavage product from the mutant protein was about 10 kDa longer than that from wild type. The interpretation of these results is that Scc1p is actually cleaved at two sites approximately 10 kDa apart. Cleavage at the more C-terminal site is highly efficient, which is why C-terminal tagged proteins cleaved only at the more N-terminal site were rarely detected.

To identify the second cleavage site, sequences within Scc1p which are similar in sequence to those surrounding the known C-terminal cleavage site were looked for. A 5 out of 7 amino acid match at position 180 found. Furthermore, the distance between this potential site and the known cleavage site is consistent with the greater length of the cleavage product generated in vitro from protein whose C-terminal site (R268) had been mutated. The matching sequence also contained a pair of arginines and therefore the more N-terminal arginine was mutated to aspartic acid. Next the effect of expressing HA tagged versions of wild type Scc1p, both single mutant proteins, and the double mutant protein from the GAL1 promoter in yeast was compared. As a host for these studies a strain was used whose endogenous Scc1p was myc tagged. Neither wild type nor either single mutant blocked cell proliferation but expression of the double mutant protein was lethal. Chromatin from cells transiently expressing these proteins was prepared and it was shown that HA tagged double mutant protein was no longer cleaved when incubated in Esp1-containing extracts while the myc tagged wild type protein was efficiently cleaved.

To investigate why cells expressing a non-cleavable Scc1p protein (the R180D R268D double mutant) cannot proliferate, centrifugal elutriation was used to isolate G1 cells from a culture growing in the absence of galactose , which were then incubated in the presence and absence of galactose (Fig. 4). In order to minimize the duration of mutant protein expression, the cells grown in the presence of galactose were transferred to glucose containing medium after most cells had replicated their DNA (at 135 min). In the absence of galactose, sister separation and dissociation from chromosomes of endogenous myc tagged Scc1p occured simultaneously, approximately 60 min after DNA replication. Transient expression of double mutant protein greatly reduced sister chromatid separation (Fig. 4b) but did not affect dissociation of endogenous myc tagged wild type protein (Fig. 4c and d). Furthermore, the mutant protein remained tightly associated with chromosomes long after the endogenous wild type protein had disappeared. Expression of the mutant protein did not greatly delay cell cycle progression and most cells underwent cytokinesis, producing progeny with low (0-0.5C) amounts of DNA and cells with less than a 2C DNA content (Fig. 4a). The dissociation from chromosomes of wild type protein on schedule shows that the lack of sister separation in cells expressing non-cleavable Scc1p is not due to a lack of Esp1 activity. Collectively, the data obtained imply that cleavage of Scc1p at one of two sites is necessary both for sister chromatid separation and for dissociation of Scc1p from chromosomes.

From the obtained results it can be concluded that cohesin directly mediates the link between sister chromatids that is established during DNA replication and is maintained until metaphase. It can be further concluded that Esp1's activation by proteolysis of Pds1 (and by as yet to be identified other mechanisms) generates an activity inside cells that cleaves the Scc1p subunit of cohesin and that this event both destroys sister chromatid cohesion and causes Scc1p and possibly other cohesin subunits to dissociate from chromosomes.

From the above results it is clear that sister chromatid separation depends on cleavage of chromosome-bound Scc1 by an Esp1-dependent proteolytic activity that appears in cells at the onset of anaphase. It was next asked whether Scc1 as an isolated protein (rather than in the chromosomal

context) can also serve as a substrate for the Esp1-dependent cleavage reaction. Fig. 5 describes the purification of recombinant Scc1 after over-expression in insect cells infected with a recombinant baculovirus. Scc1 was purified either from an asynchronously growing population of infected insect cells (Fig. 5a, lanes 1-4) or from infected insect cells that had been treated with the phosphatase inhibitor okadaic acid. Treatment with okadaic acid induces a metaphase-like state within the insect cells as a consequence of which Scc1 is obtained in a mitotically phosphorylated form (Fig. 5a, lane 5). Scc1 in yeast appears also phosphorylated in a mitosis specific manner. These Scc1 fractions, that were more than 90% pure as judged by SDS-PAGE followed by staining of the gel with Coomassie brilliant blue, were then used in the Scc1 cleavage assay as described above (Fig. 5b). Both unphosphorylated and phosphorylated purified Scc1 were cleaved in an Esp1-dependent manner in vitro, however, the efficiency of cleavage was much greater when Scc1 was in the mitotically phosphorylated state. From this experiment it was concluded that isolated Scc1 which is neither part of the cohesin compex nor bound to chromosomes is a substrate for cleavage by Esp1, at least if it is in its mitotically phosphorylated state.

It was then addressed, whether Esp1 is itself the protease that cleaves Scc1. Inhibitor studies showed that the in vitro cleavage activity could be inhibited by N-ethyl maleimide an inhibitor specific for proteases using a catalytic cysteine residue. Inspection of the amino acid sequence within the evolutionary conserved C-terminal half of Esp1 revealed that exactly one cysteine and one histidine residue are conserved in all known separin homologues. These two residues might therefore form the catalytic dyad of a new subclass of cysteine protease. When the amino acid sequences surrounding the potential catalytic dyad were further analysed, it was found that both the cysteine and the histidine residues are preceded by a sequence stretch predicted to form a hydrophobic beta sheet. Furthermore, the histidine is invariably flanked by two glycine residues and the cysteine is preceded by a glycine providing the possibility for a tight turns before or after the catalytic residues. This arrangment of histidine and a cysteine catalytic dyad residues fixed at the ends of two neighbouring strands of hydrophobic beta sheet is used in the caspase family of proteases and it seems likely that the same arrangment is used in separins like Esp1.

To provide evidence that Esp1 indeed uses these two amino acid residues histidine (amino acid position 1505) and cysteine (position 1531) as a catalytic dyad for cleaving Scc1,either of these amino acids were mutated to alanine. Both mutations completely abolished the proteolytic activity in yeast extracts after overproduction of the proteins (Fig. 6). Wild type Esp1 overexpressed to a similar level caused complete cleavage of the Scc1 substrate. It was concluded that histidine 1505 and cystein 1531 most likely form the catalytic dyad that provides Esp1 with its proteolytic activity to cleave Scc1.

Together these results provide compelling evidence that a proteolytic reaction in which Esp1 separin cleaves the cohesin Scc1 is the initiating event for sister chromatid separation at the metaphase to anaphase transition in mitosis in S. cerevisiae.

It was next asked whether the same proteolytic mechanism might act to initiate chromosome separation during the two meiotic nuclear divisions. During premeiotic DNA replication a Scc1-homolog, called Rec8, replaces Scc1 in the cohesin complex (Klein et al., 1999). Rec8, like Scc1, contains two separin recognition sites, which suggests that Esp1/separin might cleave Rec8 during meiosis to initiate meiotic chromosome separation. To test this, both separin cleavage sites within Rec8 were mutated to produce a non-cleavable version of this protein. Expression of the non-cleavable Rec8 during meiosis led to a block of the first meiotic nuclear division (Fig. 7A), indicating that cleavage of Rec8 is necessary to separate sister chromatid arms in the first meiotic division. When meiosis was followed in a yeast strain containing the *esp1-2* mutation, a temperature sensitive mutation in the ESP1 gene, a temperature dependent block of the first meiotic nuclear division was likewise observed (Fig. 7B). It was concluded that separin cleaves the cohesin Rec8 during the meiotic nuclear divisions as it cleaves Scc1 during the mitotic division.

The sequences of human homologs of budding yeast Esp1, Pds1 and Scc1 already exist in public databases. The human homologs of Esp1 and Pds1 are referred to as separin (Nagase et al., 1996; protein sequence: NCBI Acc. No. BAA11482; DNA sequence: NCBI Acc. No. D79987) and securin (Zou et al., 1999, Dominguez et al., 1998) respectively, and the human homolog of Scc1 as SCC1 (McKay et al., 1996; DNA sequence: NCBI Acc.

No. X98294; protein sequence: NCBI Acc. No. CAA 66940). In animal cells it has been shown that the majority of SCC1 dissociates from chromatin in prophase long before sister chromatids are separated in anaphase, and no cleavage of SCC1 has been observed during this process (Losada et al., 1999).

Another object of the experiments of the present invention was to test whether some SCC1 remains bound to condensed chromosomes and maintains sister chromatid cohesion until the initiation of anaphase, and to analyze whether the chromosome-bound form of SCC1 was subject to proteolytic cleavage at the onset of anaphase.

To answer these questions, the following approach was taken: Human HeLa cells were enriched in interphase by logarithmic growth and in metaphase by treatment with nocodazole, and crude chromatin and supernatant fractions were generated by differential centrifugation and analyzed for the presence of SCC1 by quantitative immunoblotting (Fig. 8). The amount of the total cellular SCC1 associated with chromatin was reduced from 56 % in logarithmically growing cells to 13 % in cells arrested in metaphase. It was concluded that most but not all SCC1 dissociates from chromatin before metaphase, consistent with the possibility that SCC1 may be required to maintain sister chromatid cohesion until the onset of anaphase.

To address whether the form of SCC1 that is associated with chromosomes in metaphase is cleaved in anaphase, HeLa cells were arrested at the onset of S-phase by double-thymidine treatment and were synchronously released into the cell cycle. Progression through the cell cycle was monitored at different time points after the release by analysis of the DNA content with fluorescence activated cell sorting (FACS) and by analyzing total cell lysates in immunoblot experiments. Figure 9 shows that a putative SCC1 cleavage product corresponding to 100 kDa was recognized by antibodies specific for the C-terminus of SCC1. Importantly, this band appeared specifically when the HeLa cells went through anaphase, as judged by FACS analysis and the disappearance of securin, cyclin B and CDC20, proteins that are known to be degraded specifically in anaphase.

To confirm that the anaphase-specific 100 kDa band is a cleavage prodcut of SCC1 and not a non-specific crossreaction of the antibodies used, the following two experiments were performed: First, antibodies specific for the N-terminus of SCC1 were raised and used to analyze the HeLa cells cycle fractions by immunoblotting. A band of 25 kDa was recognized specifically in anaphase (Fig. 9), consistent with the interpretation that SCC1 is cleaved into an C-terminal 100 kDa and a N-terminal 25 kDa fragment. Second, a HeLa cell line stably expressing mouse SCC1 fused to a myc epitope tag at the C-terminus (SCC1-myc) was analyzed by cell synchronization as above. The amount of SCC1-myc expressed in these cells is less than 10% of endogenous SCC1 and the ectopic protein is entirely incorporated into 14S cohesin complexes (Fig. 10A). In synchronized cells, antibodies to the myc epitope recognize a band of the expected size (120 kDa) that appears in anaphase with similar kinetics as the 100 and 25 kDa bands recognized by SCC1 antibodies, demonstrating unambiguously that mammalian SCC1 is cleaved in anaphase (Fig. 10B). In addition, these immunoblots revealed a second anaphase-specific fragment of SCC1-myc, suggesting that SCC1 cleavage occurs at at least two sites (Fig. 10B bottom panel).

Biochemical experiments in *Xenopus* have shown that the initiation of anaphase depends on proteolysis of securin mediated by APC^{CDC20} (Zou et al., 1999). Like budding yeast Pds1 and Esp1, securin and separin form a complex, consistent with the hypothesis that APC^{CDC20}-dependent securin proteolysis activates separin. To address whether SCC1 cleavage depends on activation of APC^{CDC20} and subsequent securin proteolysis HeLa cells were synchronized by double-thymidine treatment and released into the cell cycle in the presence of nocodazole. Nocodazole is a drug known to indirectly cause the inhibition of APC^{CDC20} and thereby to arrest cells in metaphase (reviewed by Peters, 1998). Specific antibodies to human securin were raised and it was shown that securin was not degraded under these conditions (Fig. 11). Importantly, no cleavage of SCC1 could be observed in the presence of nocodazole. The effect of the drug was reversible because release of nocodazole arrested cells into anaphase correlated with degradation of securin and formation of the SCC1 cleavage products (Fig. 12). These results suggest that SCC1 cleavage depends on activation of APC^{CDC20} and are consistent with the

hypothesis that securin degradation and subsequent separin activation are required for SCC1 cleavage.

To analyze the regulation of SCC1 cleavage further and as a first step to develop a screening assay for inhibitors of this reaction an *in vitro* assay utilizing SCC1-myc and cell cycle extracts prepared from *Xenopus* eggs was established. These extracts can be manipulated to represent either a stable interphase state in which APC^{CDC20} is inactive or a stable mitotic state in which APC^{CDC20} is active and in which sister chromatid separation can occur *in vitro* (Murray et al., 1991) When chromatin isolated from HeLa cells stably expressing SCC1-myc was incubated in *Xenopus* extracts, cleavage of SCC1 at two distinct sites could be detected in the mitotic but not in the interphase extract, further confirming that APC^{CDC20} activity is required for this event (Fig. 13). In SDS-PAGE, the cleavage products formed *in vitro* comigrated with the cleavage products formed *in vivo*, suggesting that cleavage in the extract occurs at physiologically relevant sites. Importantly, some SCC1 cleavage was also observed when chromatin-free supernatant fractions from HeLa cells were mixed with mitotic extracts (Fig. 13). This demonstrates that soluble human SCC1 can be a substrate for cleavage and thus makes the development of a simplified chromatin-free cleavage assay for drug screening purposes feasible.

To map the more N-terminal cleavage site in SCC1 a series of N- and C-terminal deletion mutants was generated and the electrophoretic mobility of the truncated proteins was compared to the mobility of the N- and C-terminal cleavage products formed *in vivo* (Fig. 14). cDNAs encoding deletion mutants were generated by polymerase chain reactions (PCR) and recombinant S^{35}-labeled proteins were generated from the PCR products by *in vitro* transcription and translation. This analysis indicated that SCC1 is cleaved between amino acid residues 169 and 183. This site contains the sequence motif $ExxR^{172}$ which is conserved in many SCC1 homologs in different species and is also found in both N-terminal cleavage sites of budding yeast Scc1. Preliminary results using the same mapping strategy indicate that the C-terminal cleavage site in SCC1 is located around amino acid residue 450 where the motif ExxR is found again.

Based on the results of the experiments of the present invention it can be concluded that separin-dependent SCC1 cleavage is a mechanism that is conserved from budding yeast to humans and that the same mechanism most likely exists in all eukaryotic organisms. The findings obtained in experiments performed with budding yeast are therefore also valid in higher eukaryotic organisms, in particular in man.

The interpretation of the data obtained in the experiments of the present invention further provides evidence that Esp1/separin itself is the protease responsible for the cleavage of Scc1p/SCC1.

From the results obtained in the experiments of the invention, it may, inter alia, be concluded that Scc1p/SCC1 is the only subunit of the cohesion comples cleaved by Esp1/separin.This does, however, not exclude the possibility that other types of proteins, for example, other cohesion proteins or proteins which regulate mitotic spindles, might also be targets/substrates of separin. One way of addressing this question is to make a version of Scc1p that has one cleavage site replaced with a site for a foreign protease (with the other cleavage site removed). An example for a convenient protease to use is TEV protease (Daugherty et al., 1989), which has a very specific cleavage site (Glu-Asn-Leu-Tyr-Phe-Gln-Gly). A strain can be constructed that contains: the SCC1 gene containing a TEV protease cleavage site, a chromosomal *cdc20-3* mutation, and the TEV protease gene under *GAL1-10* inducible control. In the presence of galactose at the restrictive temperature (when *cdc20-3* cells are arrested in metaphase due to their failure to destroy Pds1), the effect of the artificial cleavage of Scc1p on its removal from chromosomes can be assayed (as measured by its presence in sedimented chromosomal DNA fractions). Whether or not this is sufficient for sister chromatid separation can also be examined microscopically, using the CenV-GFP system (Ciosk et al., 1998; see Example 3). These experiments allow to determine whether the rest of mitosis can proceed under these conditions in the absence of separin function (note that separin is inactive in cdc20-3 mutants at the restrictive temperature due to the presence of its inhibitor Pds1). If the foreign protease triggers Scc1p's dissociation from chromatids under these circumstances and sister chromatids segregate to opposite poles of the yeast cell, it can be concluded that cleavage of Scc1 is the sole function of

separin needed for sister chromatid segregation. If however, sister chromatids fail to segregate to opposite poles of the cell despite the variant Scc1p having been removed from chromatin, then it is concluded that separin has one or more functions besides cleavage of Scc1p. A clue as to these functions can be obtained from the phenotype of these cells and this can be used to identify other potential substrates for Esp1.

The findings of the experiments of the present invention have shed the first key insight into the molecular mechanism by which eukaryotic cells separate sister chromatids. In view of the published literature, which contains no hints as to the mechanism by which sister chromatids are separated, the finding that separins act by conferring a proteolytic activity is highly surprising.

The identification of Esp1/separin as the protease responsible for Scc1/SCC1 cleavage and the identification of potential co-factors, is the prerequisite for designing assay methods that allow for finding compounds interfering with sister chromatid separation, which is the basis for novel therapeutic approaches.

In a first aspect, the invention relates to a method for identifying compounds that have the ability of modulating sister chromatid separation in plant or animal cells, characterized in that a protease with separin-like cysteine endopeptidase activity is incubated, in the presence of the substrate(s) for its proteolytic activity and optionally its co-factor(s), with test compounds and that the modulating effect of the test compounds on the proteolytic activity of the cysteine endopeptidase is determined.

By providing a method to identify compounds which exert their effect by directly modulating, in particular by inhibiting separin's proteolytic activity, i.e. by being protease inhibitors specific for separin, the present invention provides means for interfering with the mechanism of sister chromatid separation and thus a novel approach for inhibiting the proliferation of rapidly dividing animal cells, in particular tumor cells.

In the following, if not otherwise stated, the term "separin" is used as a synonym for any cysteine endopeptidase with separin-like activity, including the yeast homolog Esp1. Similarly, the term "SCC1" is not limited to the

human separin substrate, but is intended to encompass any homologous substrate of the cohesin-type.

In a first embodiment, for small scale applications, the assay of the type as described in Example 2 for the yeast components can be used to identify compounds that inhibit separin activity. Given the existence of Esp1 homologues in man, i.e. separin, it can be concluded that the separin activity plays an important role in triggering anaphase onset also in humans. Therefore, a separin-dependent cleavage assay using human separin and SCC1 instead of yeast components can be established using the principles outlined in the experiments for yeast components. Such an assay comprises, as its essential features, incubating a crude preparation of chromatin with a preparation containing a separin activity and determining SCC1 cleavage products in the presence or absence of a test substance.

In general, when setting up a screening assay, it may be useful to first perform it with yeast constituents as assay components and subsequently further develop it stepwise using the protease and/or substrate from intermediate organisms, e.g. from S. pombe or Xenopus laevis, and finally equivalent human substrates. For example, the S. pombe homologue of Scc1 (called Rad21) contains two sequences which are similar to the two known cleavage sites in Scc1, and Rad21 derived sequences may therefore be used to generate a substrate for S. pombe Esp1 (called Cut1). This process of advancing to higher organisms can be applied stepwise until a human system is attained. The cleavage site of any new substrate for human separin can be determined by purifying the cleavage product and determining the N-terminal sequences by Edman degradation as described above.

In a preferred embodiment, the method of the invention is performed on a high-throughput scale. For this embodiment the major assay components, in particular separin, are employed in recombinant form.

Depending on the desired application of the separin-inhibitor to be identified, the assay components employed may vary in terms of the species that they are derived from. In view of therapeutical applications in animals or humans, the assay components are preferably of mammalian or

human origin, in case of intended agricultural applications, the assay components are derived from plants.

Separin can be produced recombinantly according to standard methods, e.g in yeast or insect cells or in other suitable host cells, based on the sequence information in the literature or in data bases. The obtained protein can be purified by conventional biochemical fractionation from yeast cells over-producing separin or by tagging the over-produced protein with polypeptide sequences which have a special affinity for a defined ligand (affinity purification). For example, separin can be purified on nickel-agarose columns if it has been tagged with multiple histidine residues, whereas it can be purified on glutathione-agarose columns if it has been tagged with GST. Such affinity purification involves the cleavage of separin from its tag using site specific proteases or self cleaving inteins. The thus obtained recombinant protein can then be used to determine, according to known methods for assaying proteolytic activity, whether separin alone is capable of cleaving Scc1p or peptide substrates derived from it. In case that separin is alone capable of cleaving a SCC1 or a SCC1-derived peptide, an assay based on, preferably recombinant, separin as the protease and its substrate SCC1 can easily be adapted to a high throughput format by methods that are standard for other defined proteases, as described below.

The protease substrates useful in the assay may be those equivalent to or mimicking the naturally occuring substrates, e.g. crude chromatin preparations, SCC1, preferably recombinantly produced, or an SCC1 peptide that contains the proteolytic cleavage site.

Based on information about the sequence specificity of the separin proteolytic cleavage site in yeast and in man, other potential substrates for the protease can be found in other organisms, including humans, which also allows for the design of peptides derived from these substrates, which are useful as substrates in the screening assay of the invention.

In a preferred embodiment, the substrate is a peptide containing the cleavage site of the naturally occuring substrate. The sequence specificity of the proteolytic cleavage can be determined by testing a variety of different peptides. The peptide may be of natural origin, i.e. derived from

the natural SCC1, or a variant. An example for a natural peptide ist the human SCC1 peptide as set forth SEQ:ID:NO:1, or a fragment thereof that contains the separin cleavage site. Variants can be generated either by synthesising variant peptides or by mutating DNA sequences from genes encoding cohesion proteins. More specifically, other substrates for separin can be identified by searching for small DNA fragments from the yeast genome or an oligonucleotide library that can replace the normal Scc1 cleavage sites. Oligonucleotides may be inserted into a SCC1 gene (lacking both natural cleavage sites) under control of the GAL promoter on centromeric plamid. Yeast cells may be transformed with a library of such constructs and only plasmids whose modified Scc1 protein can be cleaved by the separin activity will permit growth in the presence of galactose. The peptides encoded by the positive constructs are useful as substrates for separin in the screeing assay of the invention.

With regard to the substrate, e.g. the SCC1 protein or a peptide fragment thereof, care needs to be taken that the substrate is efficiently cleaved. It has to be considered, in particular when using the yeast homolog of SCC1, that efficient cleavage appears to occur only when the substrate is in its phosphorylated state, as it is present in mitosis. Therefore, when designing a peptide substrate or when producing SCC1 recombinantly, it has to be tested whether the substrate is efficiently cleaved by separin. In case of the recombinant protein, it can be obtained in its phosphorylated form by producing it in infected insect cells that are treated with a phosphatase inhibitor, e.g. okadaic acid. This method is exemplified, for the yeast Scc1 protein, in Example 5 (method section e) and can, if necessary for other SCC1 molecules, be adapted for these molecules.

In the case that separin does not act by itself, but in cooperation with co-factors, instead of incubating SCC1 (or peptide substrates) with separin alone, they can be incubated with a mixture of separin and its co-factors. All components can be produced and purified according to standard methods as outlined above for separin.

For the high throughput format, the screening methods of the invention to identify separin inhibitors, are carried out according to assay methods known in the art for identifying protease inhibitors. Such assays are based on the detection of the cleavage products of the substrate. To achieve this,

an SCC1 peptide or protein substrate that contains cleavage sites for the separin protease is derivatized with a detectable label, e.g a radioactive or a fluorescent label. Upon cleavage of the substrate by the protease, the cleavage product can be measured. If a test substance is an inhibitor of the protease, there will be, depending on the detection system and depending on whether the test substance has an inhibiting or an activating effect, a decrease or an increase in the detectable signal.

In the high-throughput format, compounds with a modulationg effect on separin or a separin-like cysteine endopeptidase can be identified by screening test substances from compound libraries according to known assay principles, e.g. in an automated system on microtiter plates.

Recently, various assay methods for identifying protease inhibitors have been described that are amenable to automation in a high-throughput format, e.g. the radiometric method described by Cerretani et al., 1999, for hepatitis C virus NS3 protease, the method based on fluorescence quenching described by Ambrose et al., 1998, or by Taliani et al., 1996, the microtiter colorimetric assay fot the HIV-1 protease described by Stebbins and Debouck, 1997, the fluorescence polarization assay described by Levine et al., 1997 (reviewed by Jolley, 1996), the method using immobilized peptide substrates described by Singh et al., 1996, the assay used for studying the inhibition of cathepsin G, using biotinylated and cysteine-modified peptides described by Brown et al., 1994. A further example for a suitable assay is based on the phenomenonon of fluorescence resonance energy transfer (FRET), as described by Gershkovich et al., 1996 or by Matayoshi et al., 1990. Additional examples for assays that may be used in the present invention for a high-throughput screening method to identify inhibitors of separin activity were described by Gray et al., 1994, Murray et al., 1993, Sarubbi et al., 1991.

Fluorescent or radioactive labels and the other reagents for carrying out the enzymatic reaction on a high-throughput scale are commercially available and can be employed according to supplier's instructions (e.g. Molecular Probes, Wallac). The specific assay design depends on various parameters, e.g. on the size of the substrate used. In the the case of using a short peptide, the fluorescence quenching or the fluorescence resonance energy transfer methods are examples for suitable assay technologies.

The fluorescence quenching (Resonance Energy Transfer „RET") assay relies on synthetic substrates which are capable of direct, continuous signal generation that is proportional to the extent of substrate hydrolysis. The substrate peptide carries a fluorescent donor near one end and an acceptor near the other end. The fluorescence of the substrate is initially quenched by intramolecular RET between donor and acceptor. Upon cleavage of the substrate by the protease the cleavage products are released from RET quenching and the a fluorescence proportional to the amount of cleaved substrate can be detected.

An assay of this type may be carried out as follows: the solution of the labeled substrate (e.g. the peptide labeled with 4-[[4'-(dimethylamino)phenyl]azo]benzoic acid (DABCYL) at the one end and with 5-[(2'-aminoethyl)amino]naphtalenesulfonic acid (EDANS) at the other end or labeled with benzyloxycarbonyl at the one end and with 4-aminomethylcoumarin at the other end) in assay buffer is pipetted into each well of black 96-well microtiter plates. After addition of the test substances in the defined concentration, the separin solution is added to the wells. After incubation under conditions and for a period of time sufficient for the proteolytic cleavage reaction, e.g. for 1 hour at room temperature, the fluorescence is measured in a fluorometer at the excitation wavelength, e.g. at 340 nm, and at the emission wavelength, e.g. at 485 nm.

In the case of using the FRET assay, the following commercially availabe labeling pairs are suitable for the method of the invention: Europium (Eu) and Allophycocyanin (APC), Eu and Cy5, Eu and PE (Wallac, Turku, Finland).

The compounds identified in the above methods, which are also subject of the invention, have the ability to interfere with sister chromatid separation by modulating the proteolytic activity of a separin-like cysteine endopeptidase.

In a preferred embodiment, the compounds of the invention are inhibitors of a separin-like cysteine endopeptidase.

Preferably, the compounds are specific inhibitors of separin.

The present invention also relates to compounds which act as inhibitors of separin for use in human therapy, in particular cancer therapy.

In a further aspect, the invention relates to a pharmaceutical composition which contains, as the active ingredient, one or more compounds which interfere with or modulate sister chromatid separation by inhibiting the proteolytic activity of separin.

The present invention also encompasses inhibitors of any protease that is recognized to be a separin-like protease because of its sequence similarity to separins; i.e all proteases in which amino acid sequences surrounding the catalytic dyad are more similar to separins than to any class of protease currently known.

In a preferred embodiment, the invention comprises pharmaceutically active compounds and their use in therapy, which are small chemical molecules that have been identified as separin inhibitors in the screening method of the invention.

Alternatively, the separin inhibitors may be biological molecules, e.g. peptides or peptide-derived molecules like pepidomimetics.

Proteases from the caspase family, to which separin is likely to belong, have been shown to be good targets for irreversible binding and inhibition by peptide derived inhibitors (Nicholson et al., 1995; Faleiro et al., 1997). In principle, the approach described for the caspase inhibitors, which act as "recognition site peptides" by being modified to contain an aldehyde, halogenomethyl or acyloxymethyl group at the cleavage position, can be adapted to irreversibly bind to and inhibit the active site cysteine residue in separin. Inhibitory peptide derivatives of this type can be the starting point for rational inhibitor design, e. g. derivatives of the peptide spanning the amino acid sequence at the protease recognition site in SCC1 or another separin substrate. An example for a peptide to be considered for such design is the peptide derived from human SCC1, MDDREIMREGSAFEDDDM (SEQ:ID:NO:1), which contains the separin cleavage site, or a mutation or fragment thereof. The inhibitor design can also be aided by obtaining structural information about the catalytic domain of Esp1 using x-ray crystallography. Initially the structure of the Esp1

catalytic domain can also be modelled onto the already known structures of two members of the caspase family of proteases.

The efficacy of compounds identified as separin inhibitors can be tested for *in vivo* efficacy either on yeast cells or in mammalian cells. Effective compounds should block (or at least in some way interfere with) sister chromatid separation, which can be measured, e.g. by using CenV-GFP in yeast, as described by Ciosk et al., 1998, or standard cytological techniques in mammalian cells. Effective compounds should be either cytostatic or cytotoxic. Substances whose potential for therapeutic use has been confirmed in such secondary screen can be further tested for their effect on tumor cells. To test the inhibition of tumor cell proliferation, primary human tumor cells are incubated with the compound identified in the screen and the inhibition of tumor cell proliferation is tested by conventional methods, e.g. bromo-desoxy-uridine or ^3H incorporation. Compounds that exhibit an anti-proliferative effect in these assays may be further tested in tumor animal models and used for the therapy of tumors.

Toxicity and therapeutic efficacy of the compounds identified as drug candidates by the method of the invention can be determined by standard pharmaceutical procedures, which include conducting cell culture and animal experiments to determine the IC_{50}, LD_50, the ED_{50}. The data obtained are used for determining the human dose range, which will also depend on the dosage form (tablets, capsules, aerosol sprays, ampules, etc.) and the administration route (oral, buccal, nasal, paterental or rectal). A pharmaceutical composition containing the compound as the active ingredient can be formulated in conventional manner using one or more physologically active carriers and excipients. Methods for making such formulations can be found in manuals, e.g. "Remington Pharmaceutical Sciences".

Influencing the process of sister chromatid separation may be also beneficial in preventing birth defects caused by missegration of chromosomes in human meioses. For example, since cases of human aneuploidy such as Down's syndrome may be caused by premature separation of sister chromatids (Griffin, 1996), the use of a drug that inhibits separin activity might be able to reduce precocious sister separation and thereby the incidence of aneuploidy in human fetuses.

Thus, in a further aspect, the invention relates to separin inhibitors for the prevention of birth defects caused by missegration of chromosomes in human meioses.

Separin inhibitors may also be useful in applications which aim at the deliberate polyploidisation of plant cells for crop development. In yeast, it has been shown that inhibition of separin activity prevents chromosome separation without blocking cell cycle progression and therefore gives rise to cells with increased ploidy. Inhibitors that block separin's protease activity could therefore be used to increase the ploidy of any eukaryotic cell, including all plant cells. Increasing the ploidy of plant cells is useful for 1) producing larger plants, 2) for increasing the ploidy of breeding stocks, and 3) for generating fertile hybrids.

Therefore, the present invention relates, in a further aspect, to separin inhibitors for the treatment of plant cells for increasing their ploidy.

To identify separin inhibitors that are useful for the above-mentioned agricultural purposes, the screening method of the invention can be easily adapted by employing plant components, i.e. a plant separin and a plant homolog of SCC1. Sequence homologs of plant separin and SCC1 are present in databases, e.g. of the Arabidopsis thaliana genome.

Separin inhibitors which impair sister chromatid separation may also be used in cytological analyses of chromosomes, for example, in medical diagnoses of chromosome structure.

Brief description of the figures:

Fig. 1: Chromosome association of Scc1p in G1 is Esp1-dependent

Fig. 2: In vitro assay for Scc1p cleavage and dissociation from chromatin

Fig. 3: Detection of the Scc1p cleavage product in vivo in cells passing sychronously throught the metaphase to anaphase transition

Fig. 4: Expression of a non-cleavable variant of Scc1p prevents Scc1p dissociation from chromosomes and sister chromatid separation in vivo

Fig. 5: Purified Scc1 is a substrate for the Esp1-dependent cleavage reaction

Fig. 6: Mutations in the putative catalytic dyad of the Esp1 protease domain abolish cleavage activity

Fig. 7: Separin cleavage of the cohesin Rec8 is necessary during meiotic nuclear divisions

Fig. 8. Association of human SCC1 with chromatin

Fig. 9: Human SCC1 is cleaved in mitosis

Fig. 10: Ectopically expressed SCC1-myc is incorporated into the cohesin complex and is cleaved in mitosis

Fig.11: Human SCC1 is not cleaved in human cells arrested in metaphase by nocodazole treatment

Fig. 12: Human SCC1 is cleaved in anaphase

Fig. 13. Human SCC1-myc is cleaved in vitro

Fig. 14: Mapping of the N-terminal cleavage site of human SCC1

If not otherwise stated, the following materials and methods were used in the experiments of the present invention

a) Yeast plasmids and strains:

The Scc1p coding sequence (Saccharomyces Genome Database YDL003W) was cloned under control of the *GAL1-10* promoter in a YIplac128 derived vector (Gietz and Sugino, 1988), and under its own promoter into YCplac111 (Gietz and Sugino, 1988) using the polymerase chain reaction (PCR). DNA fragments encoding multiple HA and myc epitopes were inserted into restriction sites introduced by PCR at the N- and C-termini of SCC1. Site directed mutagenesis was performed by PCR using primers containing the desired nucleotide changes. The validity of all constructs was verified by nucleotide sequencing.

All strains used were derivatives of W303 (*HMLa HMRa ho ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*). Epitope tags at the endogenous Scc1p were generated by a PCR one-step tagging method (Michaelis et al., 1997). A strain overexpressing Esp1p from the *GAL1-10* promoter was described (Ciosk et al., 1998) and crossed to a strain containing the *esp1-1* mutation (McGrew et al., 1992). A strain expressing the sole source of Cdc20p under control of the *GAL1-10* promoter was described in (Lim et al., 1998). To visualize sister chromatids a Tet repressor-GFP fusion protein is synthesized in the cells that binds to a cluster of Tet operator sequences integrated at the *URA3* locus close to the centromere of chromosome V as described in (Michaelis et al., 1997).

All meiotic yeast strains used in Example 7 are derivatives of the rapidly sporulating SK1. The Rec8 431/453 mutant (E428R R431E R453E) was generated by site-specific mutagenic PCR of a REC8 wild-type integrative plasmid. This YIplac128-derived plasmid (Gietz and Sugino, 1988) contains the REC8 gene and promoter and 3 HA epitope sequences at the C-terminus. This plasmid was integrated at the rec8::KanMX4 locus by transformation with the MluI-linearized plasmid.

The esp1-2 allele (McGrew et al., 1992) was recovered from strain K8493 using a gap repair strategy as described by Guthrie and Fink, 1991. The recovered allele was transferred into SK1 by transformation and 5-FOA counter-selection (Guthrie and Fink, 1991). The resultant temperature-sensitive strain was diploidized by transformation with plasmid c1743 containing the HO gene. For sporulation experiments, strains were first

streaked from the -80°C stock onto a YEPGlycerol plate and grown for 60 hours at 25°C. A single colony was patched onto YEPD and grown for 48 hours at 25°C. The patch of cells was innoculated into liquid YEPD and grown for 8 hours to stationary phase. The culture was washed with YEPA and grown overnight in YEPA. Cells were washed with 2% Potassium Acetate and then incubated for 14-24 hours in the same medium. Samples were taken every two hours and fixed with 70% ethanol for visualizing nuclei by DAPI staining.

b) Yeast cell growth and cell cycle experiments

Cells were grown in complete medium (Rose et al., 1990) at 25°C if not otherwise stated. Strains expressing Cdc20p, Esp1p, or Scc1p from the *GAL1-10* promoter were grown in complete medium containing 2% Raffinose as carbon source. The *GAL1-10* promoter was induced by adding 2% galactose. A G1 like arrest was achieved by adding 1 µg/ml of the pheromone alpha factor to the medium. For a metaphase arrest, 15 µg/ml nocodazole was added with 1% DMSO. Metaphase arrest due to Cdc20p depletion was obtained in cells with the sole source of Cdc20p under control of the *GAL1-10* promoter. A logarithmically growing culture in complete medium containing raffinose and galactose was filtered, washed with medium containing Raffinose only, and resuspended in the same medium. For release from the arrest 2% Galactose was added back to the culture.

c) In vitro assay for yeast Esp1p activity

A crude Triton X-100 insoluble chromatin preparation was obtained from yeast cells as described (Liang and Stillman, 1997). The pelleted chromatin was resuspended in yeast cell extracts that had been prepared similar to the supernatant fraction of the chromatin preparation. One tenth volume of an ATP regenerating system was added (50 mM HEPES/KOH pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 600 mM creatin phosphate, 1.5 mg/ml phophocreatin kinase, 1 mM DTT, 10% glycerol). Reactions were incubated for 10 min at 25°C with shaking and stopped on ice. The chromatin fraction was separated again from the supernatant by centrifugation, and resuspended in buffer EBX (Liang and Stillman, 1997). Equivalent aliquots of supernatant and chromatin pellet were analysed by

SDS-PAGE and Western blotting. Scc1-HA was detected with the anti-HA monoclonal antibody 16B12 (Boehringer Mannheim).

Since overexpression of Esp1p from the *GAL1-10* promoter is toxic to cells, extracts with overproduced Esp1p were prepared 2 h after induction with galactose of a culture pregrown in medium containing raffinose only.

d) Protein sequencing of the yeast Scc1p cleavage site

The C-terminal Scc1p cleavage fragment was isolated from cells that contained Scc1p tagged with 18 tandem myc epitopes at the C-terminus. A Cdc20 arrest/release strategy was employed to obtain cells containing a high portion of Scc1p in the cleaved form. Protein extract of 5×10^9 cells was prepared by breakage with glass beads 15 min after release from the metaphase arrest. myc-epitope tagged protein was immunoprecipitated with 20 mg anti-myc 9E11 monoclonal antibody under denaturing conditions and resolved on SDS-PAGE next to size markers. Proteins were transferred to a PVDF membrane and stained with Coomassie Brillant Blue R250. N-terminal sequencing of the band corresponding to the Scc1p cleavage fragment yielded the amino acid sequence RLGESIM (Scc1p amino acids 269 onwards).

e) Purification of yeast Scc1 expressed in baculovirus infected insect cells

The Scc1 coding sequence was cloned into the baculovirus transfer vector pFastBac1 (Gibco Life Technologies). At the C-terminus a FLAG epitope tag was added followed by a cassette containing the yeast VMA intein and a chitin binding domain (New England Biolabs). Recombinant baculoviruses were obtained following the manufacturer's instructions. HiFive insect cells (Invitrogen) were grown in monolayers to confluency and infected at an multiplicity of infection of 2 with the recombinant baculovirus. To obtain metaphase-like phosphorylation 0.1 □M Okadaic acid was added 40 hours after infection. 43 hours after infection cells were harvested. Cytoplasmic and nuclear extracts were obtained as described (Cai et al., 1996). Scc1 was purified from the combined extracts by chitin affinity chromatography according to the manufacturer's protocols, and further purified by two subsequent ion exchange chromatography steps on a MonoQ column (Amersham Pharmacia).

f) Mutations in the yeast Esp1 catalytic dyad

Esp1 was overexpressed as described in a). The conserved residues histidine 1505 and cysteine 1531 that form the putative catalytic dyad of the Esp1 protease were changed to alanine using a PCR based mutation scheme. The mutant proteins were expressed from the GAL promoter in yeast and assayed for there cleavage activity as described under c).

g) Other yeast methods

Analysis of DNA content was performed as described (Epstein and Cross, 1992) on a Becton Dickinson FACScan, chromosome spreads were as described (Michaelis et al., 1997), photo micrographs were taken with a Photometrics CCD camera mounted on a Zeiss Axiophot microscope.

In vitro translation of Pds1p was performed in reticulocyte lysate using the TNT system (Promega).

h) Human cells

HeLa cells were cultivated in DMEM supplemented with 10% FCS at 37 °C and 5% CO_2.

In some experiments HeLa cells stably expressing mouse SCC1 fused to 9 myc ecpitopes at its C-terminus were used.

i) Human cell cycle experiments

For cell cycle synchronization, a double-thymidine treatment was used. HeLa cells were first treated with 2 mM thymidine for 18 h. Subsequently, cells were washed with PBS, fresh medium was added and the cells were grown for another 8 h. Then the cells were treated again with 2 mM thymidine for 18 h, subsequently washed and incubated in fresh medium. Samples were taken at different time points. Samples were splitted and used for FACscan analysis and for immunoblotting. Cell extracts were made with glass-teflon potters in ice cold buffer containing 50 mM Tris pH 7.7, 100 mM NaCl, 20 mM b-glycerophosphate, 5 mM $MgCl_2$, 1 mM NaF, 0.1-0.2% Triton X-100, 10% Glycerol, 1 mM DTT and protease inhibitors).

In some experiments logarithmically growing HeLa cells were treated with 330 nM nocodazole dissolved in 0.1 % DMSO for 18 hours, or cells synchronized by double-thymidine treatment were released into nocodazole-containing medium for different periods of time. In control experiments 0.1 % DMSO was added without nocodazole.

For SDS-PAGE, equal amounts of total extract was loaded (usually 50 □g protein per sample). For western blotting the following antibodies were used: Monoclonal mouse-anti-separin a C-terminal fragment of human separin was expressed in pET28Vector, His-tagged protein was purified and used for immunization); rabbit-anti-SCC1 (N-terminal or C-terminal peptides of human SCC1 were coupled to KLH and used for immunization); rabbit anti-securin (human securin was expressed in pTrcHis2 vector, His/myc-tagged protein was purified and used for immunization). All antibodies were affinity purified. CDC27, CDC20 and proteasome antibodies have been described (Gieffers et al., 1999). Mouse-anti-Cyclin B1 (#SC-245) were from Santa Cruz Biotechnology, USA. Rabbit-anti-Cyclin A (#06-138) and rabbit-anti -phopho-Histone H3 (#06-570) were from Upstate Biotechnology, USA. Rabbit-anti-myc-epitope antibodies (CM-100) were from Gramsch, Germany.

j) In vitro cleavage of SCC1-myc

Whole cell extract (containing 250 µg protein) from nocodazole-arrested HeLa cells ectopically expressing mouse SCC1-myc was separated by centrifugation into chromatin and supernatant fractions. Either 12.5 ul of the supernatant fraction or the chromatin pellet (resuspended in 5 ul of buffer containing 0.005 % Triton X100, 20 mM Hepes pH 7.7, 20 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$) were incubated with 25 µl of either interphase or mitotic Xenopus egg extract at room temperature. At different time points samples were taken, separated by SDS-PAGE and analyzed by immunoblotting with anti-myc antibodies. Xenopus extracts were prepared as described (Murray, 1991).

k) Mapping of the N-terminal cleavage site of human SCC1

For generating truncated versions of the human SCC1 cDNA, polymerase chain reactions (PCRs) were used. For N-terminal deletions different 5'-

primers containing T7 promotor regions, a start codon and appropriate SCC1 sequences were used. For C-terminal deletions different 3'-primers with appropriate SCC1 sequences and a stop-codon were used. The obtained PCR fragments were transribed and translated in the presence of ^{35}S-methionine in reticulocyte lysate in vitro (TNT system, Promega). The in vitro translated products were separated by SDS-PAGE and immunoblotted with C- or N-terminal specific SCC1 antibodies.

Example 1

Chromosome association of yeast Scc1p in G1 is Esp1-dependent

A) Cells, wild type for *ESP1* or containing the *esp1-1* mutation, with an unmodified endogenous copy of Scc1 and a second myc-tagged copy under the control of the *GAL* promoter were arrested with the mating pheromone alpha factor for 120 min. All cells had then arrested in the G1 phase of the cell cycle (time point 0 of the experiment). The FACScan profile of the DNA content is shown, demonstrating that all cells stayed arrested during the following 120 min time course of the experiment. Scc1myc was induced for 60 min by adding 2% galactose, then cells were transferred to medium containing 2% glucose to repress Scc1myc expression (Fig. 1A).

B) Expression of Scc1myc was seen by whole cell in situ hybridization (open circles), and chromosome binding of Scc1myc was observed using chromosome spreads (filled squares). The percentage of cells positive for Scc1myc expression and that had Scc1 myc bound to chromosomes is shown in the graphs (Fig. 1B).

Example 2

In vitro assay for yeast Scc1p cleavage and dissociation from chromatin

Chromatin was prepared from a strain containing Scc1p tagged with HA epitopes that was arrested in metaphase with nocodazol. The proteins in the chromatin preparation were resolved by SDS-PAGE and Scc1-HA was detected by western blotting (Fig. 2, lane 1). This chromatin preparation

was resuspended in the indicated extracts, with or without addition of in vitro translation products as indicated. Incubation was for 10 min at 25°C, after which the chromatin was separated again from the supernatant by centrifugation. Aliquots of the supernatant fraction and the chromatin fraction of each reaction were analysed.

Example 3

Detection of the yeast Scc1p cleavage product in vivo in cells passing sychronously throught the metaphase to anaphase transition

The strain used expressed Cdc20p under the control of the GAL promoter as the only source of Cdc20p. Scc1p was tagged with HA epitopes, and sister chromatids were visualized by tetR-GFP bound to tetO sequences inserted at the centromere of chromosome V. Cells were arrested at metaphase by depleting the cells of Cdc20p in medium lacking galactose for 120 min. Then 2% galactose was added to induce Cdc20p synthesis.

A) The FACscan profile of the time course is shown in Fig. 3A.

B) Budding (Fig. 3b, filled squares) was scored, all cells arrested after 120 min with large buds and cytokinesis happend for most cells between 30 min and 45 min after induction of Cdc20p synthesis. Scc1-HA bound to chromosomes was seen on chromosome spreads (Fig. 3B, open circles) in most cells in the arrest, and Scc1-HA disappeared from chromosomes within 15 min after release. The percentage of cells with separated sister chromatids as seen as the occurrence of two separated GFP dots in one cell body is presented (Fig. 3B, filled triangles).

C) Examples of cells in the arrest at 120 min and 15 min after release. The synchronous separation of sister chromatids is visible as separating GFP dots (Fig. 3C).

D) Western blot analysis of whole cell extracts at the indicated time points. The cleavage fragment of Scc1-HA is apparent at 135 min short after the release from the metaphase block into anaphase (Fig. 3D).

Example 4

Expression of a non-cleavable variant of yeast Scc1p prevents Scc1p dissociation from chromosomes and sister chromatid separation in vivo

A) FACscan profile of the DNA content as unbudded G1 cells were released into the cell cycle either with or without the induction of the Scc1RR-DD mutant (Fig. 4A).

B) Budding index without (Fig. 4B, open squares) or with (Fig. 4B, open triangles) induction of Scc1RR-DD. Sister chromatid separation in the cells was monitored by counting the percentage of cells containing two separated GFP dots (Fig. 4B, filled squares for the control culture not expressing Scc1RR-DD, and filled triangles for the culture expressing Scc1RR-DD).

C) Scc1p chromosomse association was measured on chromosome spreads. The endogenous wild type Scc1myc is shown for the control cells (open squares) and cells expressing Scc1RR-DD (Fig. 4c, open triangles). The Scc1RR-DD was HA tagged and detected on chromosome spreads of the induced culture (Fig. 4C, filled triangles).

D) Examples of chromosome spreads of both cultures at 150 min in metaphase and at 180 min when most cells of the control culture had undergone anaphase. The DNA was stained with DAPI, Scc1myc was detected with a rabbit-anti-myc antiserum and anti-rabbit-Cy5 conjugated secondary antibody, Scc1RR-DD-HA was detected with the mouse monoclonal antibody 16B12 and anti-mouse-Cy3 conjugated secondary antibody. Sister chromatids of centromere V were visualized by the GFP dots (Fig. 4D).

Example 5

Purified yeast Scc1 is a substrate for Esp1-dependent cleavage

Fig. 5A: Purification of Scc1 from baculovirus-infected insect cells. SDS-PAGE followed by staining with Coomassie brilliant blue R250 of control HiFive whole cell extract (lane 1), whole cell extract after infection with the virus expressing Scc1 (lane 2), the eluate from the chitin affinity column (lane 3), and the pooled fraction of the second MonoQ chromatographic

step (lane 4). Scc1, purified in a similar way, but containing metaphase-like phosphorylation, is shown in lane 5.

Fig. 5B: Cleavage assay using purified Scc1. Purified Scc1 in both the unphosphorylated and the metaphase-like phosphorylated form was used as a substrate in a cleavage assay. The cell extract containing Esp1 was as in Figure 2, but was used mixed in different ratios with the control extract to obtain a titration of the Esp1 activity. Scc1 was detected by Western blotting with the anti-FLAG monoclonal antibody M2 (Sigma).

Example 6

Mutation of the catalytic dyad in yeast Esp1 abolishes its cleavage activity. Wildtype Esp1 and mutants H1505A and C1531A were overexpressed in yeast, tagged with a FLAG epitope for detection. Fig 6A: Western blot of cell extracts showing that the two mutant Esp1 proteins were expressed as stable proteins to similar levels as the wild type protein. Fig. 6B: The associated Scc1 cleavage activity was assayed as in Example 2.

Example 7

Preventing cleavage of the meiotic cohesion protein Rec8 by mutations in its cleavage sites or by an esp1 mutation inhibits meiotic nuclear divisions

Fig. 7A: Diploid yeast strains either wild type for Rec8 or expressing Rec8 with both cleavage sites mutated were sporulated. The percentage of cells containing either one nucleus, two nuclei, or three or four nuclei is depicted throughout the time course of the experiment.

Fig. 7B: A diploid yeast strain homozygous for the esp1-2 mutation was sporulated at 25°C or 35°C. The percentage of cells containing either one nucleus, two nuclei, or three or four nuclei is depicted.

Example 8

Association of human SCC1 with chromatin

Chromatin and supernatant fractions were prepared from HeLa cells that were either growing logarithmically (log) or were arrested in metaphase with nocodazole (noc). Proteins from equivalent aliquots of these fractions

were separated by SDS-PAGE and analyzed for the presence of SCC1 by immunoblotting using radiolabeled antibodies (Fig. 8, top panel), The intensities of the SCC1 bands were quantitated (Fig. 8, bottom panel).

Example 9

Human SCC1 is cleaved in mitosis

HeLa cells were arrested at the onset of S-Phase by a double-thymidine treatment and were synchronously released into the cell cycle. Samples were taken at the indicated time points. Cells were analyzed for their DNA content by FACscan (Fig. 9A) and by SDS-PAGE and immunoblotting of whole cell extracts using the indicated antibodies (Fig. 9B). The phosphorylation-dependent electrophoretic mobility shift of the APC subunit CDC27 was used as a marker for mitotic entry. The disapperance of cyclin A was used as a marker for metaphase, and the disappearance of CDC20, securin and cyclin B as a marker for anaphase. Proteasome levels were determined as a loading control. The arrows in panels 3 and 4 of Fig. 9B from the top indicate 100 and 25 kDa bands that are recognized by antibodies that are specific for the C- and the N-terminus of SCC1, respectively.

Example 10

Ectopically expressed SCC1-myc is incorporated into the cohesin complex and is cleaved in mitosis

Fig. 10A: An extract of logarithmically growing HeLa cells stably expressing SCC1-myc was separated by 5-20% sucrose density gradient centrifugation. Proteins from each gradient fraction were analyzed by SDS-PAGE and immunoblotting using antibodies to SCC1, the myc epitope and against the human cohesin subunit SA1. The position of 9S and 14S cohesin complexes is indicated. Both endogenous SCC1 and ectopically expressed SCC1-myc are exclusively found in the fractions containing the 14S cohesin complex.

Fig. 10B: HeLa cells stably expressing SCC1-myc were arrested by double-thymidine treatment and synchronously released into the cell cycle. Samples were taken at the indicated time points and whole cell extracts

were analyzed by SDS-PAGE and immunoblotting using antibodies agaist the myc epitope and the C-terminus of SCC1. SCC1 cleavage products are indicated by arrows. Two exposures of the myc immunoblot are shown to reveal a second SCC1-myc cleavage product of higher electrophoretic mobility that can only be detected in prolonged exposures (bottom panel).

Example 11

Human SCC1 is not cleaved in human cells arrested in metaphase by nocodazole treatment

HeLa cells stably expressing SCC1-myc were synchronized by double-thymidine treatment and were released into the cell cycle in the presence of either DMSO (results shown in Fig. 11A and C) or nocodazole dissolved in DMSO (results shown in Fig. 11B and D). At indicated time points samples were taken. Cells were analyzed by FACsan (Fig. 11A and B) and by immunoblotting of whole cell extracts using the indicated antibodies (Fig. 11 C and D). Antibodies specific for the C-terminus of SCC1 were used. In addition to the antibodies used in Example 9, an antibody specific for mitotically phosphorylated histone H3 (PhosphoH3) was used as a mitotic marker.

Example 12

SCC1 is cleaved in anaphase

HeLa cells stably expressing SCC1-myc were arrested in metaphase with nocodazole and synchronously released into anaphase. Samples were taken at the indicated time points. Cells were analyzed by FACscan (Fig. 12A) and by immunoblotting of whole cell extracts using the indicated antibodies (Fig. 12B). Antibodies specific for the C-terminus of SCC1 were used.

Example 13

SCC1-myc is cleaved in vitro

Chromatin and supernatant fractions were isolated by differential centrifugation from nocodazole-arrested HeLa cells stably expressing SCC1-myc. The chromatin fraction was incubated in mitotic and interphase

Xenopus egg extracts and the supernatant fraction in mitotic Xenopus egg extract. Samples were taken at the time points indicated in Fig. 13 and analyzed by SDS-PAGE and immunoblotting using antibodies to the myc epitope. Whole cell extracts from SCC1-myc expressing HeLa cells in S-phase or in anaphase (obtained by release from a double-thymidine arrest for 6 and 11.5 hours, respectively) were analyzed side by side. Cleavage products of SCC1-myc that are specifically formed in mitotic Xenopus extrcats are indicated by arrows.

Example 14

Mapping of the N-terminal cleavage site of human SCC1

Truncated versions of the human SCC1 cDNA were generated by PCR and transcribed and translated in vitro. The ^{35}S-labeled in vitro translation products (^{35}S-IVT) were analyzed by immunoblotting with SCC1 antibodies (top panels) and by phosphorimaging (bottom panels). N-terminal deletion mutants were analyzed by immunoblotting with antibodies specific for the C-terminus of SCC1 (Fig. 14, left panels) and C-terminal deletion mutants were analyzed with antibodies specific for the N-terminus of SCC1 (Fig. 14, right panels). Extracts from SCC1-myc expressing HeLa cells in anaphase or in G1 (obtained by release from a double-thymidine arrest) were analyzed side by side. The SCC1 cleavage products detected in HeLa extracts are indicated by arrows.

References

Ambrose WP, et al., (1998), *Anal Biochem*, Oct 15, **263**(2): 150-7

Brown AM, et al., (1994), *Anal Biochem*, Feb 15, **217**(1): 139-47

Cai J, et al., (1996): Reconstitution of human replication factor C from its five subunits in baculovirus-infected insect cells. *Proc. Natl. Acad. Sci. USA* **93**, 12896-12901

Cerretani M, et al., (1999), *Anal Biochem,* Jan 15, **266**(2): 192-7

Ciosk R, Zachariae W, Michaelis C, Shevchenko A, Mann M, and Nasmyth K (1998): An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* **93**, 1067-1076

Cohen-Fix O, Peters J-M, Kirschner MW, and Koshland D (1996): Anaphase initiation is Sacharomyces cervisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev* **10**, 3081-3093

Dougherty WG, Cary SM and Dawn Parks T (1989): Molecular Genetic Analysis of a Plant Virus Polyprotein Cleavage Site: A Model. Virology 171, 356-364

Dominguez A, Ramos-Morales F, Romero F, Rios RM, Dreyfus F, Tortolero M and Pintor-Toro JA (1998): hpttg, a human homologue of rat pttg, is overexpressed in hematopoietic neoplasms. Evidence for a transcrptional activation function of hPTTG. *Oncogene* **17**: 2187-2193

Epstein CB, and Cross FR (1992) CLB5: a novel cyclin from budding yeast with a role in S-phase. *Genes Dev.* **6**, 1695-1706

Faleiro L, Kobayashi R, Fearnhead H, and Lazebnik Y (1997): Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. *EMBO J.* **16**, 2271-2281

Funabiki H, et al., (1996): Cut2 proteolysis required for sister-chromatid separation in fission yeast. *Nature* **381**, 438-441

Gershkovich, A.A. and Kholodovych, V.V. (1996), J Biochem Biophys Meth 33, 135

Gietz RD and Sugino A (1988): New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**, 527-534

Gray NM, et al., (1994), *Anal Biochem*, **216**(1): 89-96

Guthrie, C. and Fink, G.R, editors (1991): Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, Volume 194, Academic Press, Inc., USA

Hayden JH, Bowser SS, and Rieder CL (1990): Kinetochores capture astral microtubules during chromosome attachment to the mitotic spindle: direct visualization in live newt lung cells. *J. Cell Biol.* **3**, 1039-1045

Jolley, M.E. (1996), J Biomol Screening 1, 33

Kramer ER, Gieffers C, Hölzl G, Hengstschläger M and Peters JM (1998): Activation of the human anaphase-promoting complex by proteins of the CDC10/Fizzy family. *Current Biology* **8**: 1207-1210

Kerrebrock AW, Moore DP, Wu JS, and Orr-Weaver TL (1995): Mei-S332, a Drosophila protein required for sister chromatid cohesion, can localize to meiotic centromere regions. *Cell* **83**, 247-256

Klein F, et al., (1999): A central role for the cohesins in sister chromatid cohesion , formation of axial elements, and recombination during yeast meiosis. *Cell* **98**, 91-103

Levine LM, et al., (1997), *Anal Biochem*, Apr 5, **247**(1): 83-8

Liang C, and Stillman B (1997): Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. *Genes Dev* **11**, 3375-3386

Lim HH, Goh P-Y, and Surana U (1998): Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast. *Curr. Biol.* **8**, 231-234

Losada A, Hirano M, and Hirano T (1998): Identification of Xenopus SMC protein complexes required for sister chromatid cohesion. *Genes Dev* **12**, 1003-1012

Matayoshi, E.D. (1990), Science 247, 954

McKay MJ, et al., (1996): Sequence conservation of the rad21 Schizosaccharomyces pompe DNA double-strand break repair gene in human and mouse. *Genomics* **36**, 305-315

McGrew JT, Goetsch L, Byers B, and Baum P (1992): Requirement for ESP1 in the nuclear division of S. cerevisiae. *Mol. Biol. Cell* **3**, 1443-1454

Merdes A, and De Mey J (1990): The mechanism of kinetochore-spindle attachment and polewards movement analyzed in PtK2 cells at the prophase-prometaphase transition. *Eur. J. Cell Biol.* **53**, 313-325

Michaelis C, Ciosk R, and Nasmyth K (1997): Cohesins: Chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**, 35-45

Miyazaki WY, and Orr-Weaver TL (1994): Sister chromatid cohesion in mitosis and meiosis. *Annu. Rev. Genet.* **28**, 167-187

Moore DP, Page AW, Tang TTL, Kerrebrock AW, and Orr-Weaver TL (1998): The cohesion protein Mei-S322 localizes to condensed meiotic and mitotic centromeres until sister chromatids separate. *J. Cell Biol.* **140**, 1003-1012

Murray AW (1991): Cell cycle extracts. Methods In cell biology **36**, 581-605

Murray MG, et al., (1993), *Gene*, Nov 30, **134**(1):123-8

Nagase T, Seki N, Ishikawa K, Tanaka A, and Nomura N (1996): Prediction of the coding sequences of unidentified human genes. V. The coding

sequences of 40 new genes (KIAA0161-KIAA0200) deduced by analysis of cDNA clones from human cell line KG-1. *DNA Res* **3**, 17-24

Nicholson DW, et al., (1995): Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**, 37-43

Peters J-M (1998): SCF and APC the Yin and Yan of cell cycle regulated proteolysis. *Curr. Op. Cell Biol.* **10**, 759-768

Remington's Pharmacuetical Sciences, 1980, Mack Publ. Co. Easton, PA, Osol (ed.)

Rieder CL, and Salmon ED (1998): The vertebrate cell kinetochore and its roles during mitosis. *Trends Cell. Biol.* **8**, 310-318

Rose MD, Winston F, and Hieter P (1990): Laboratory course manual for methods in Yeast Genetics (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York)

Sarubbi E, et al., (1991), *FEBS Lett*, Feb 25, **279**(2): 265-9

Singh J, et al., (1996), *Bioorg Med Chem*, **4**(5): 639-43

Stebbins J. and Debouck C., (1997), *Anal Biochem*, Jun 1, **248**(2): 246-50

Taliani M, et al., (1996), *Anal Biochem*, Aug 15, **240**(1):60-7

Uhlmann F, and Nasmyth K (1998): Cohesion between sister chromatids must be established during DNA replication. *Curr. Biol.* **8**, 1095-1101

Zou H, McGerry TJ, Bernal T, Kirschner MW (1999): Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science* **285**:418-422

Claims

1. A method for identifying compounds that have the ability of modulating sister chromatid separation in plant or animal cells, characterized in that a protease with separin-like cysteine endopeptidase activity is incubated, in the presence of the substrate(s) for its proteolytic activity and optionally its co-factor(s), with test compounds and that the modulating effect of the test compounds on the proteolytic activity of the cysteine endopeptidase is determined.

2. The method of claim 1, wherein said protease is recombinant.

3. The method of claim 1 or 2, wherein said protease is human separin.

4. The method of claim 1, wherein said substrate is a protein recombinantly produced in baculovirus in the presence of a phosphatase inhibitor.

5. The method of claim 1, wherein the substrate is human SCC1 oder a fragment or variant thereof.

6. The method of claim 5, wherein the substrate is a peptide with the amino acid sequence as set forth in SEQ:ID:NO:1 or a cleavable fragment or variant thereof.

7. The method of claim 1 or 2, wherein the protease is a plant separin.

8. The method of any one of claims 1 to 7, wherein the substrate carries a label which generates a detectable signal proportional to the amount of the cleavage product of the proteolytic activity, and wherein the signal is measured in the presence and in the absence of the test compound.

9. The method of claim 8, wherein the label is fluorescent.

10. Inhibitors of a protease with separin-like cysteine endopeptidase activity for human therapy.

11. Inhibitors of human separin for human cancer therapy.

12. The use of inhibitors of a protease with separin-like cysteine endopeptidase activity for the preparation of a medicament for the treatment of cancer.

13. Inhibitors of human separin for the prevention of birth defects caused by missegration of chromosomes in meioses.

14. The use of inhibitors of a protease with separin-like cysteine endopeptidase activity for the preparation of a medicament for the prevention of birth defects caused by missegration of chromosomes in meioses.

15. Pharmaceutical composition, containing as active ingredient an inhibitor of human separin.

16. Inhibitors of plant separin for increasing the ploidy of plant cells.

Fig. 1 a

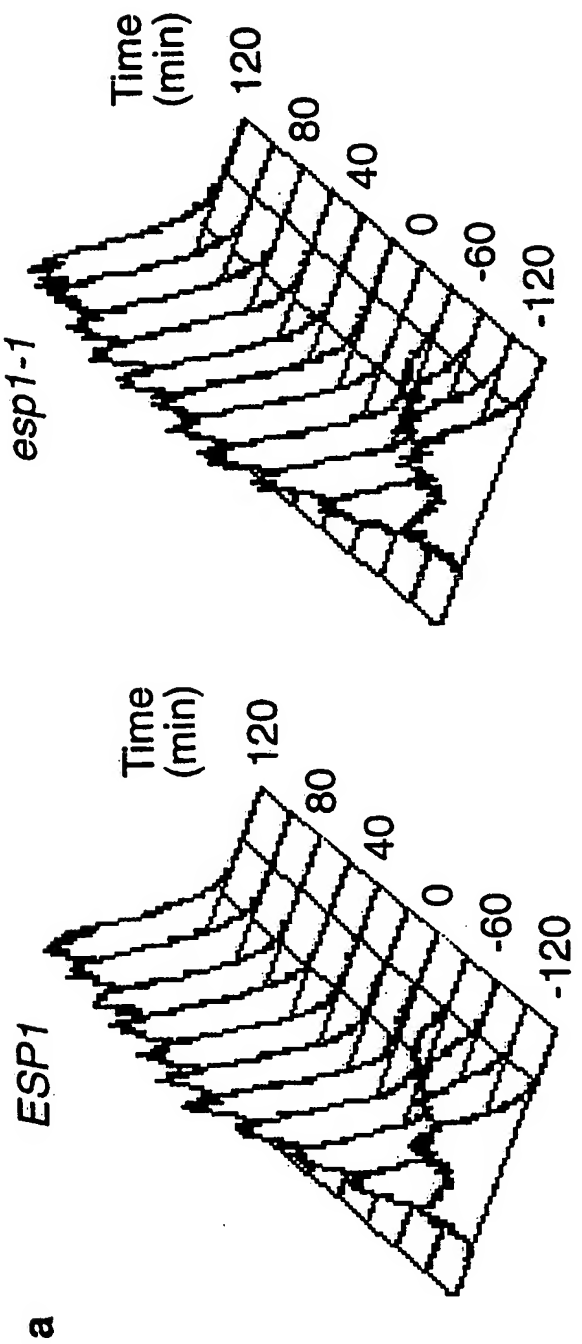
Time (min)
120
80
40
0
-60
-120
esp1-1
Time (min)
120
80
40
0
-60
-120
ESP1
a

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Fig. 1 b

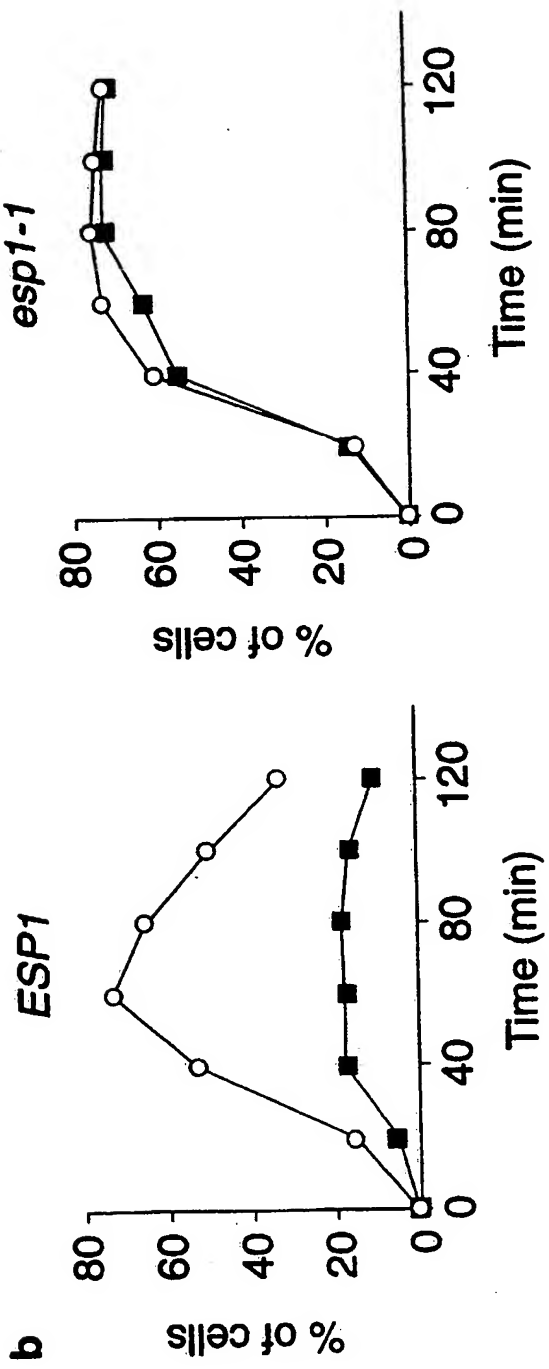
esp1-1
% of cells
0
20
40
60
80
0
40
80
120
Time (min)
ESP1
% of cells
0
20
40
60
80
0
40
80
120
Time (min)
b

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Fig. 2

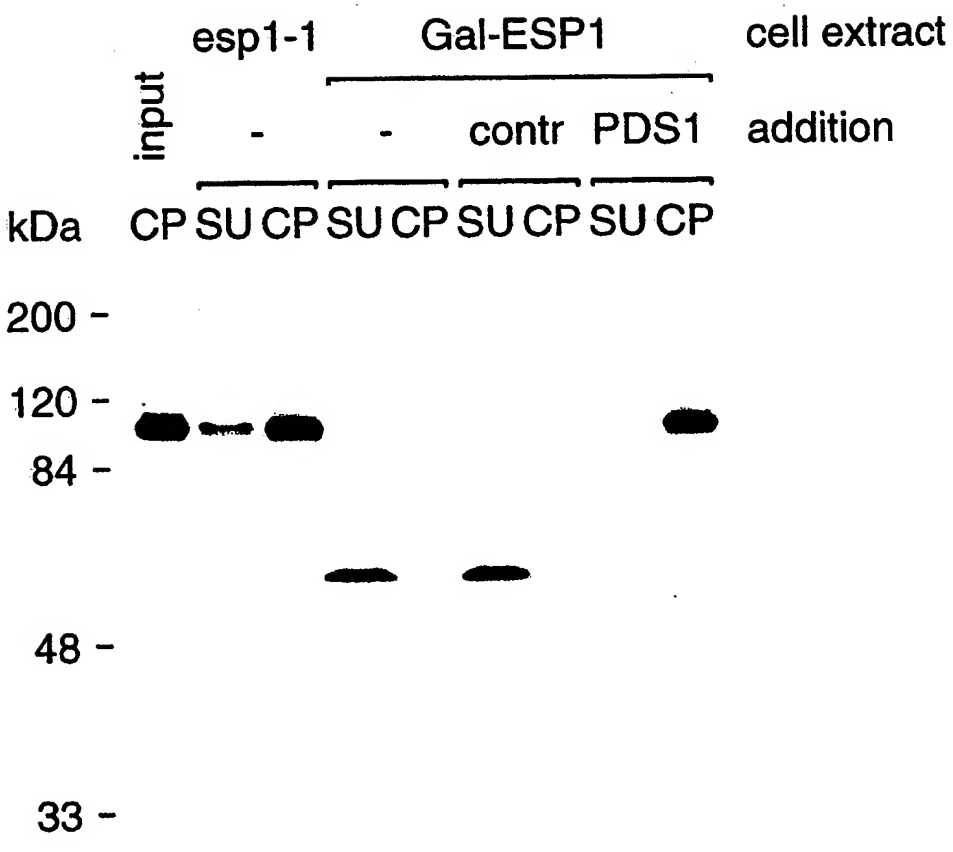
esp1-1
Gal-ESP1
cell extract
input
-
-
contr
PDS1
addition
kDa
CP SU CP SU CP SU CP SU CP
200 -
120 -
84 -
48 -
33 -

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Fig. 3 a, b

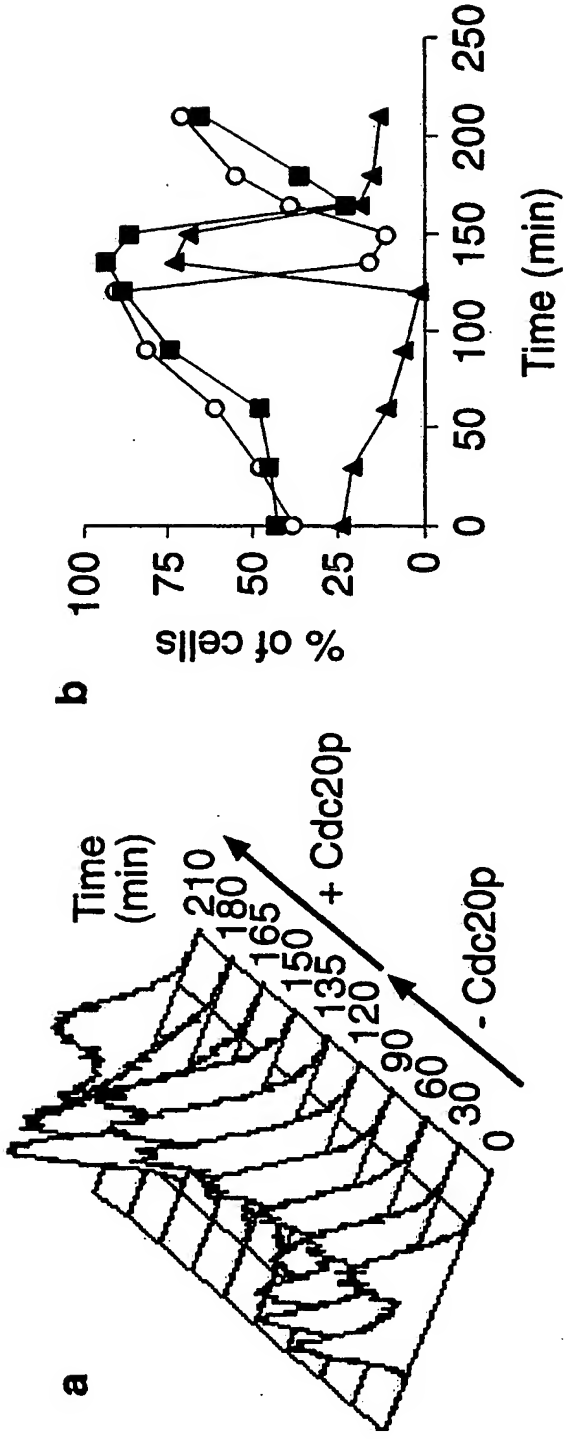
b
% of cells
100
75
50
25
0
0
50
100
150
200
250
Time (min)
a
Time (min)
210
180
165
150
135
120
90
60
30
0
+ Cdc20p
- Cdc20p

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Fig. 3 c, d

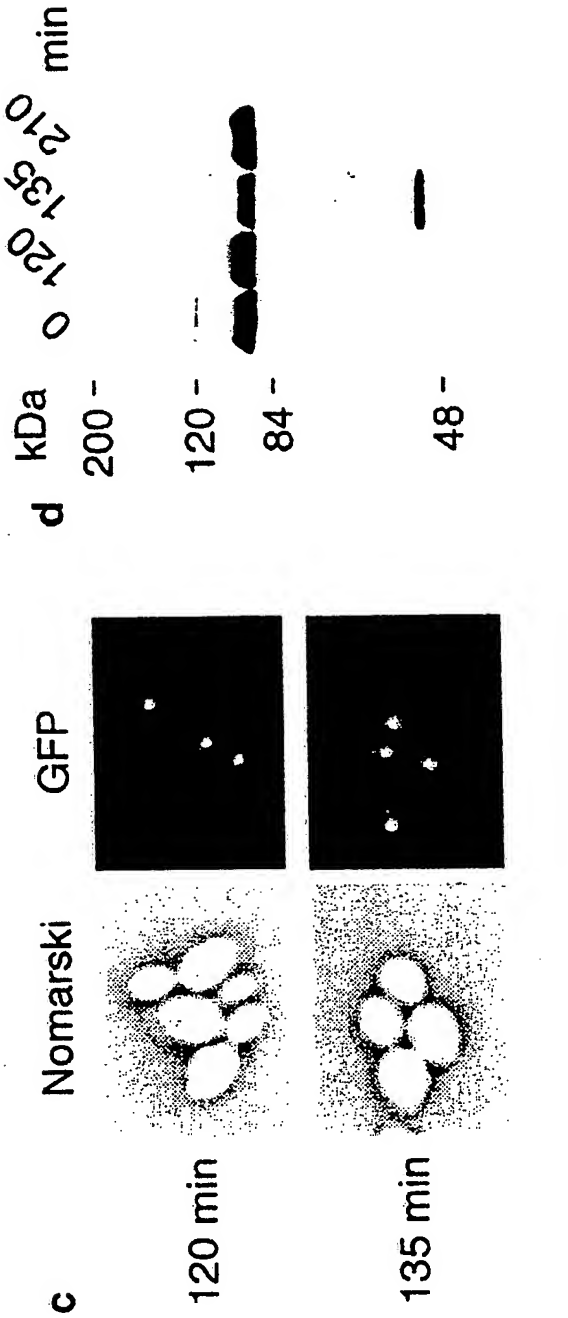
d
kDa
200 -
120 -
84 -
48 -
0
120
135
210
min
c
Nomarski
GFP
120 min
135 min

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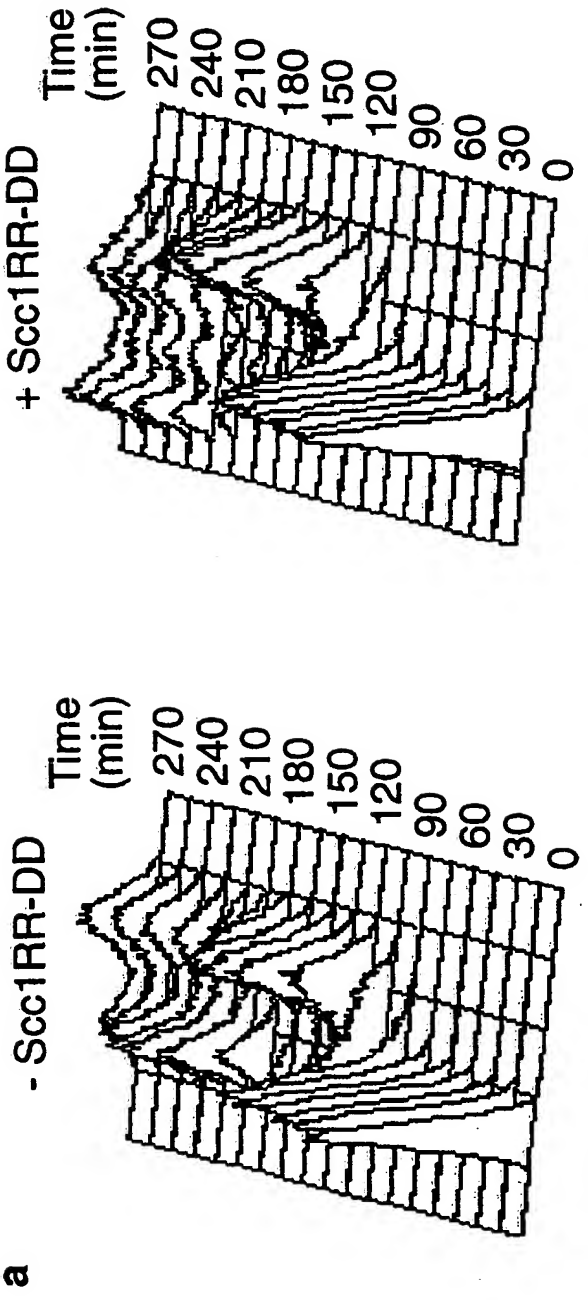
Fig. 4 a


+ Scc1RR-DD
Time (min)
270
240
210
180
150
120
90
60
30
0
- Scc1RR-DD
Time (min)
270
240
210
180
150
120
90
60
30
0

a

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Fig. 4 b, c

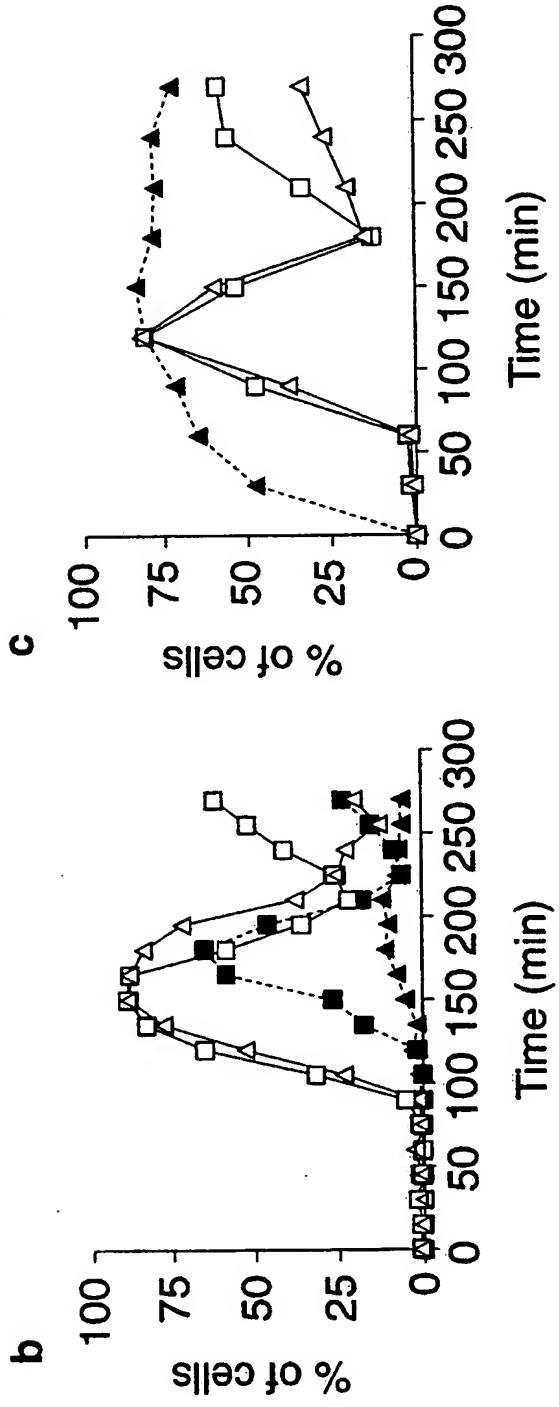
c
% of cells
100
75
50
25
0
0
50
100
150
200
250
300
Time (min)
b
% of cells
100
75
50
25
0
0
50
100
150
200
250
300
Time (min)

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Fig. 4 d

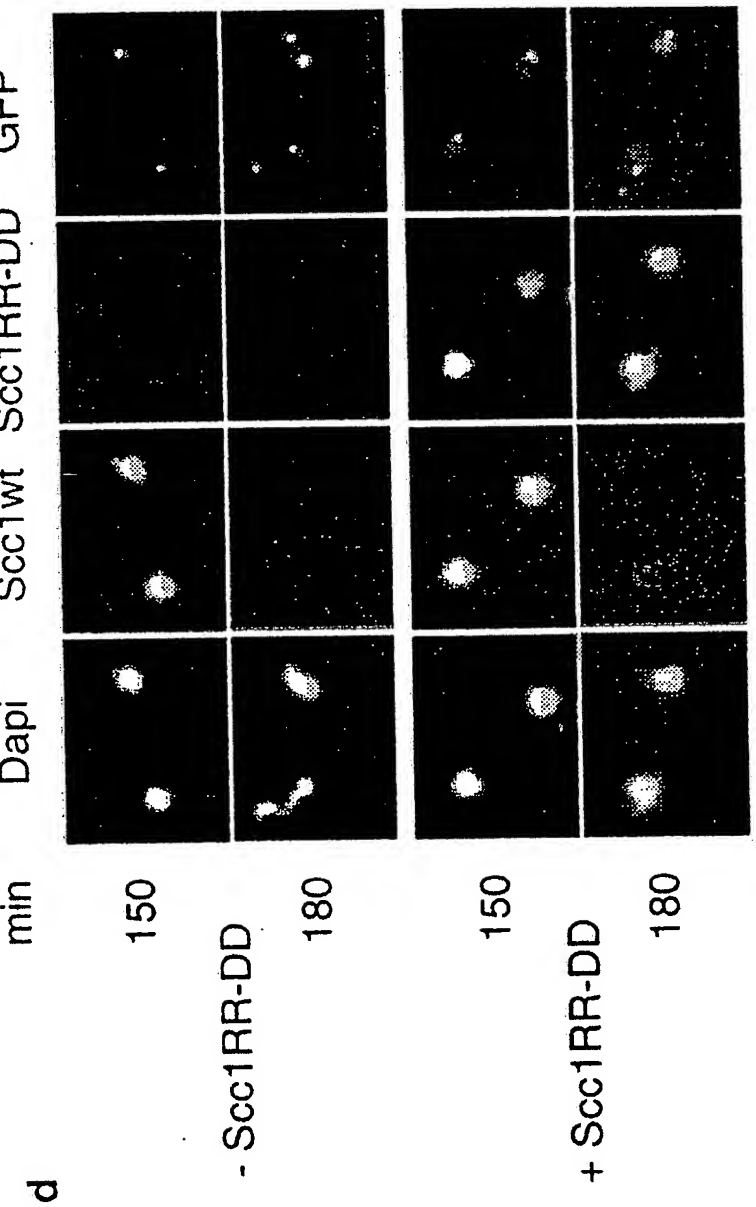
d
min
Dapi
Scc1wt
Scc1RR-DD
GFP
150
180
- Scc1RR-DD
150
180
+ Scc1RR-DD

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Fig. 5

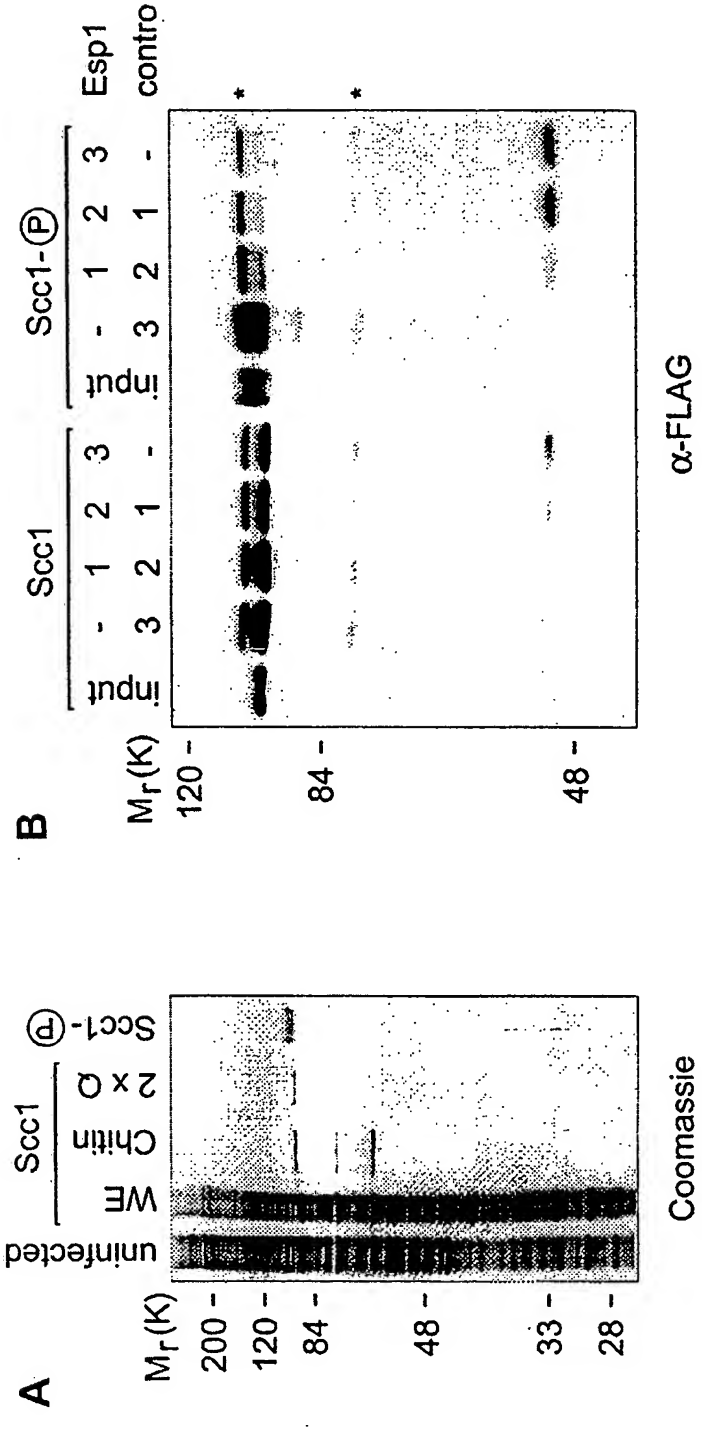
A
Mr(K)
200
120
84
48
33
28
uninfected
Scc1
WE
Chitin
2 x Ø
Scc1-Ⓟ
Coomassie
B
Mr(K)
120
84
48
Scc1
input
-
3
1
2
1
2
3
Scc1-Ⓟ
input
-
3
1
2
1
2
3
-
Esp1
contro
*
*
α-FLAG

Fig. 6

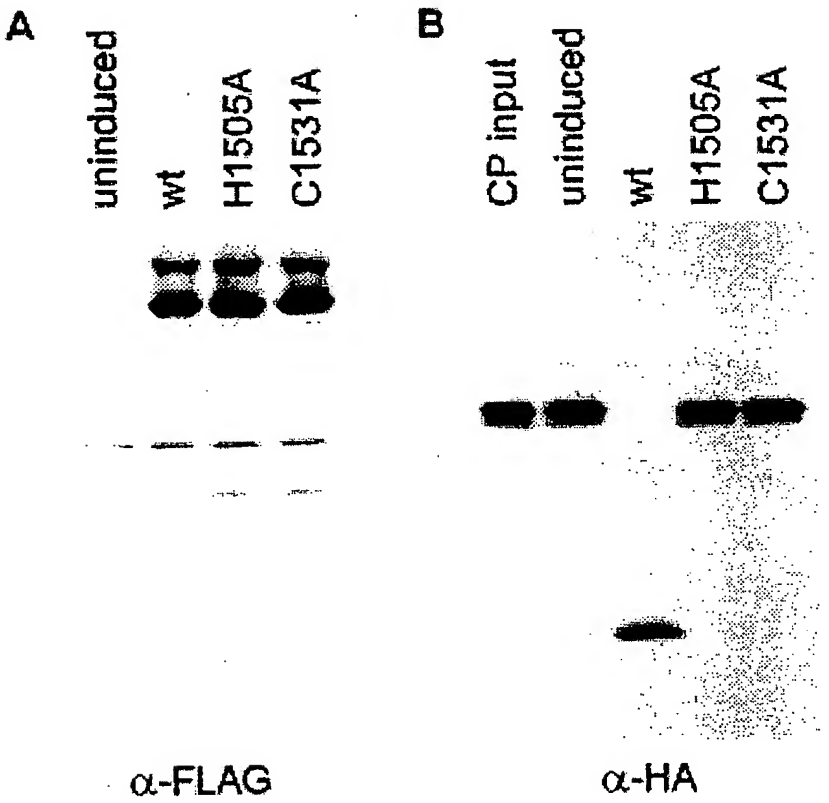
A
uninduced
wt
H1505A
C1531A
α-FLAG
B
CP input
uninduced
wt
H1505A
C1531A
α-HA

Fig. 7

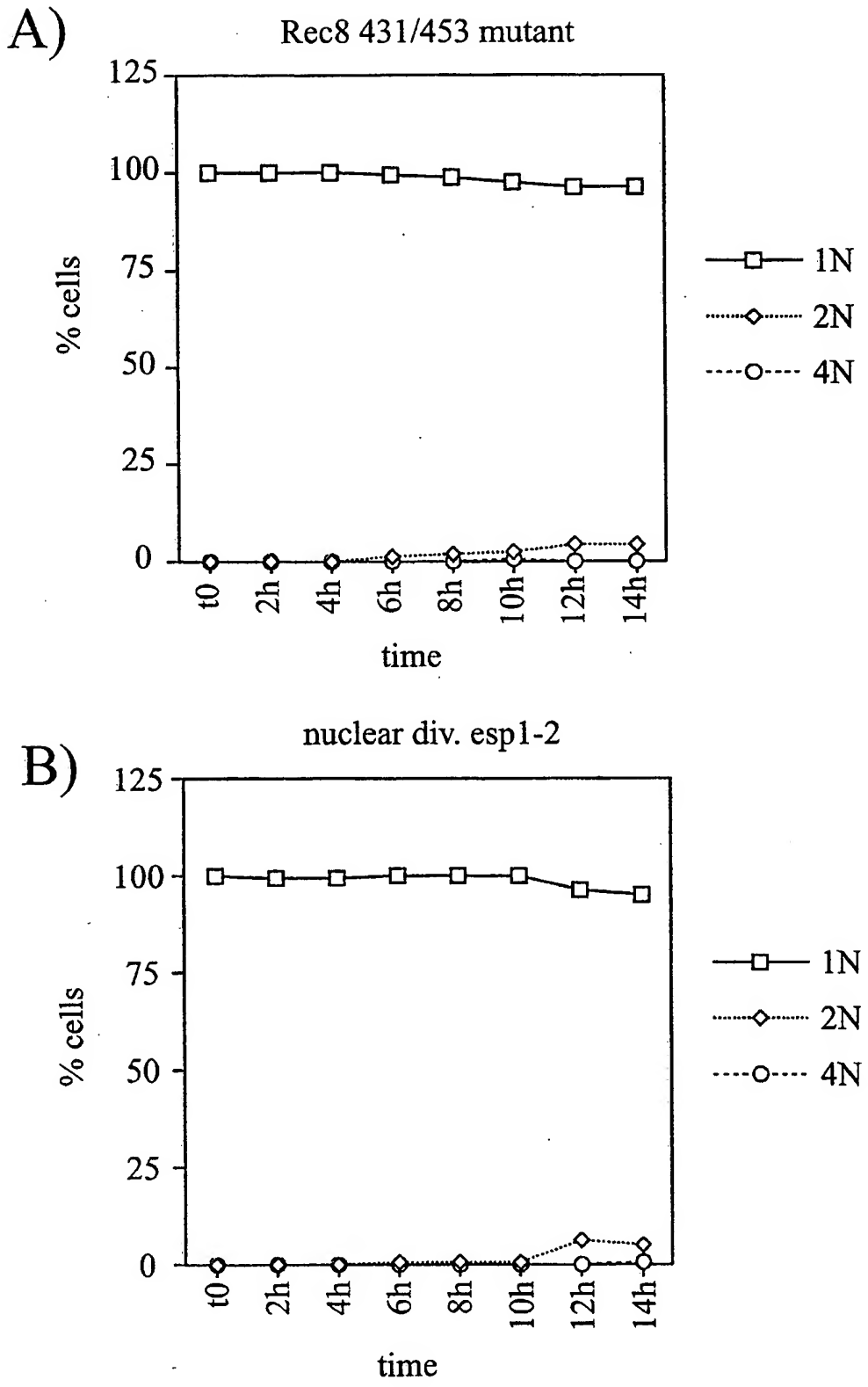
A)
Rec8 431/453 mutant
125
100
75
50
25
0
% cells
t0
2h
4h
6h
8h
10h
12h
14h
time
1N
2N
4N
B)
nuclear div. esp1-2
125
100
75
50
25
0
% cells
t0
2h
4h
6h
8h
10h
12h
14h
time
1N
2N
4N

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Fig. 8

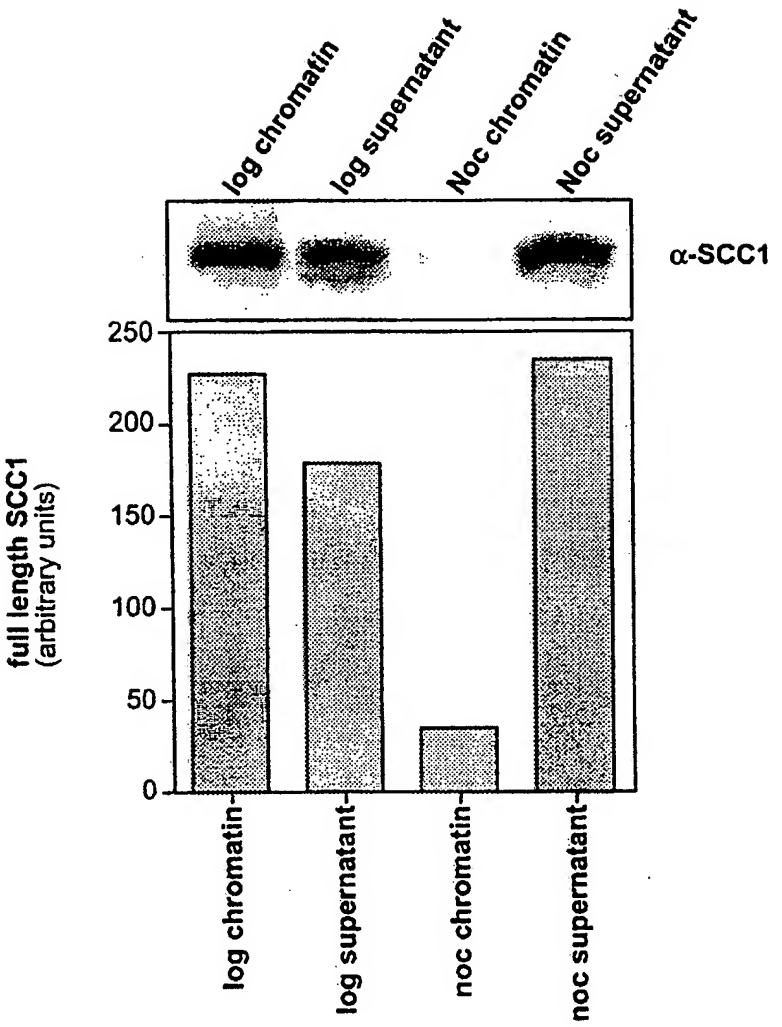
log chromatin
log supernatant
Noc chromatin
Noc supernatant
α-SCC1
full length SCC1
(arbitrary units)
250
200
150
100
50
0
log chromatin
log supernatant
noc chromatin
noc supernatant

Fig. 9 a

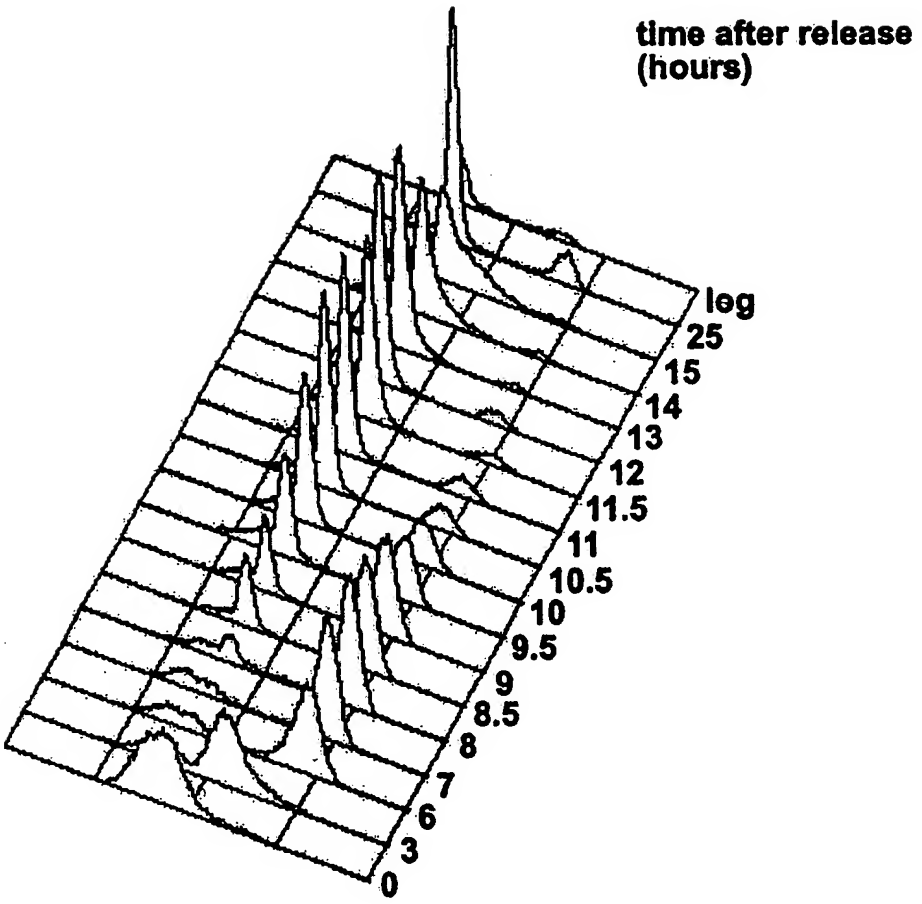
time after release
(hours)
log
25
15
14
13
12
11.5
11
10.5
10
9.5
9
8.5
8
7
6
3
0

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Fig. 9 b

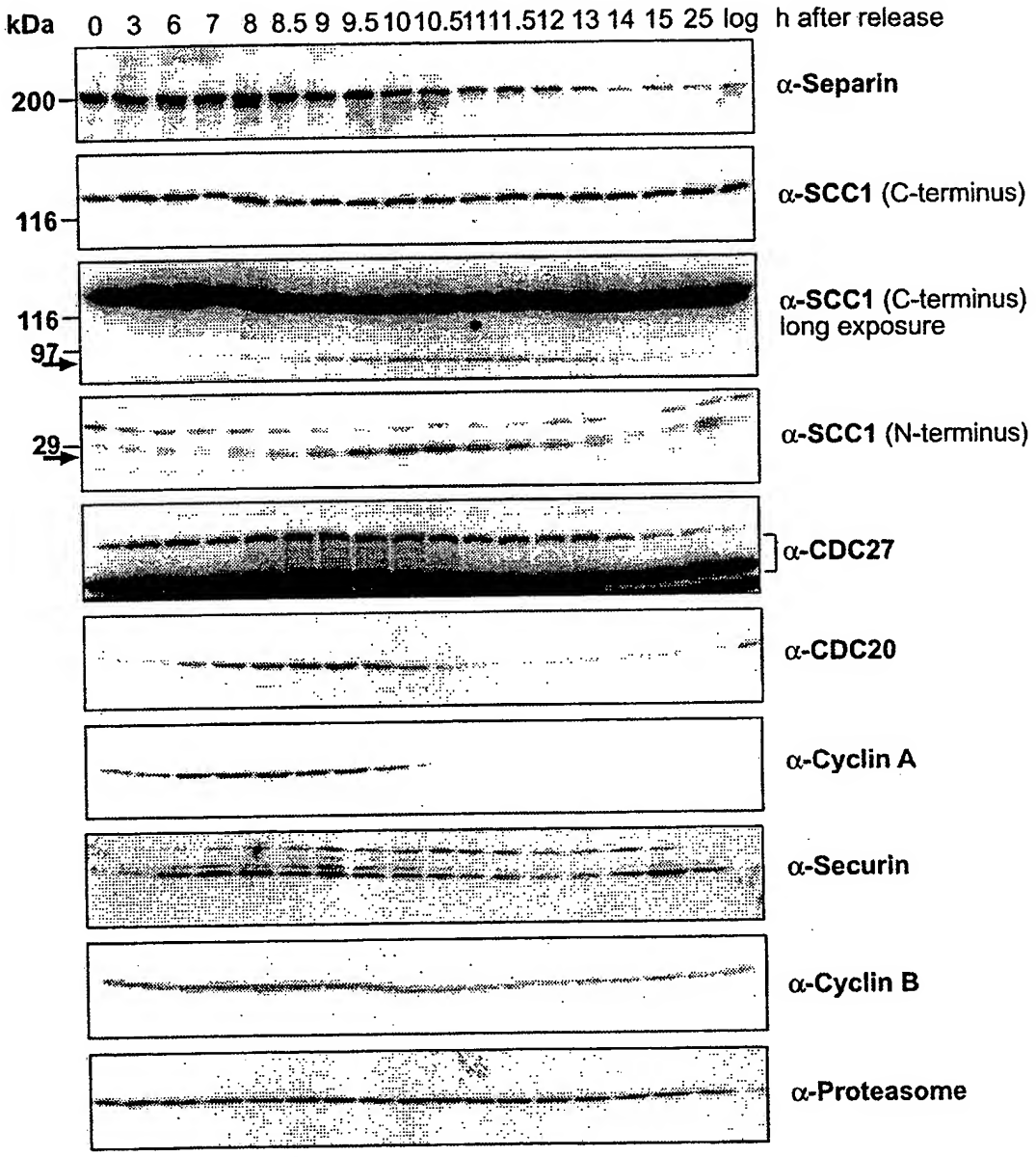
kDa
0 3 6 7 8 8.5 9 9.5 10 10.5 11 11.5 12 13 14 15 25 log h after release
200
116
116
97
29
α-Separin
α-SCC1 (C-terminus)
α-SCC1 (C-terminus)
long exposure
α-SCC1 (N-terminus)
α-CDC27
α-CDC20
α-Cyclin A
α-Securin
α-Cyclin B
α-Proteasome

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Fig. 10 a

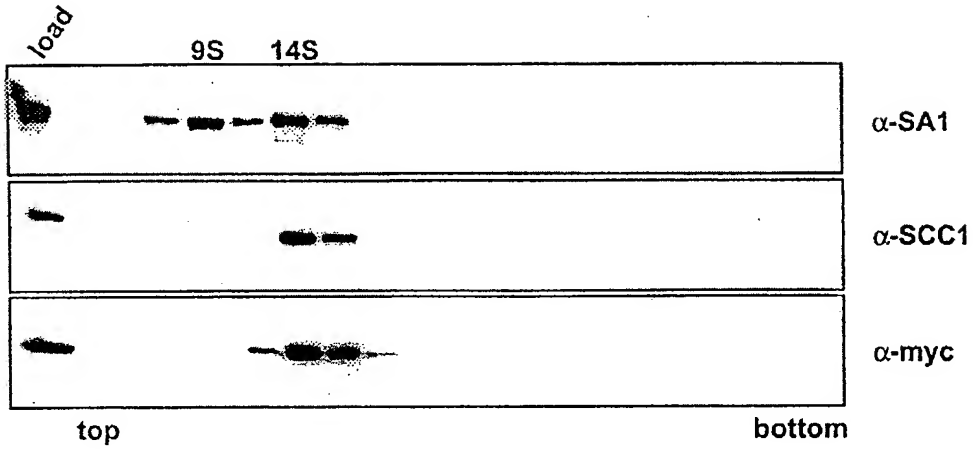

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Fig. 10 b

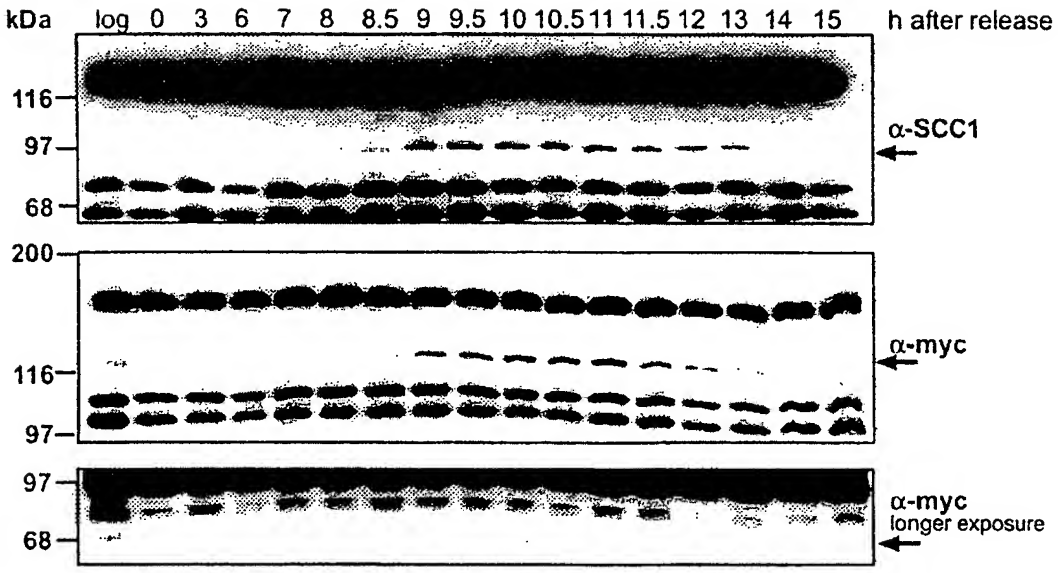
kDa
log 0 3 6 7 8 8.5 9 9.5 10 10.5 11 11.5 12 13 14 15
h after release
116
97
68
α-SCC1
200
116
97
α-myc
97
68
α-myc
longer exposure

Fig. 11 a

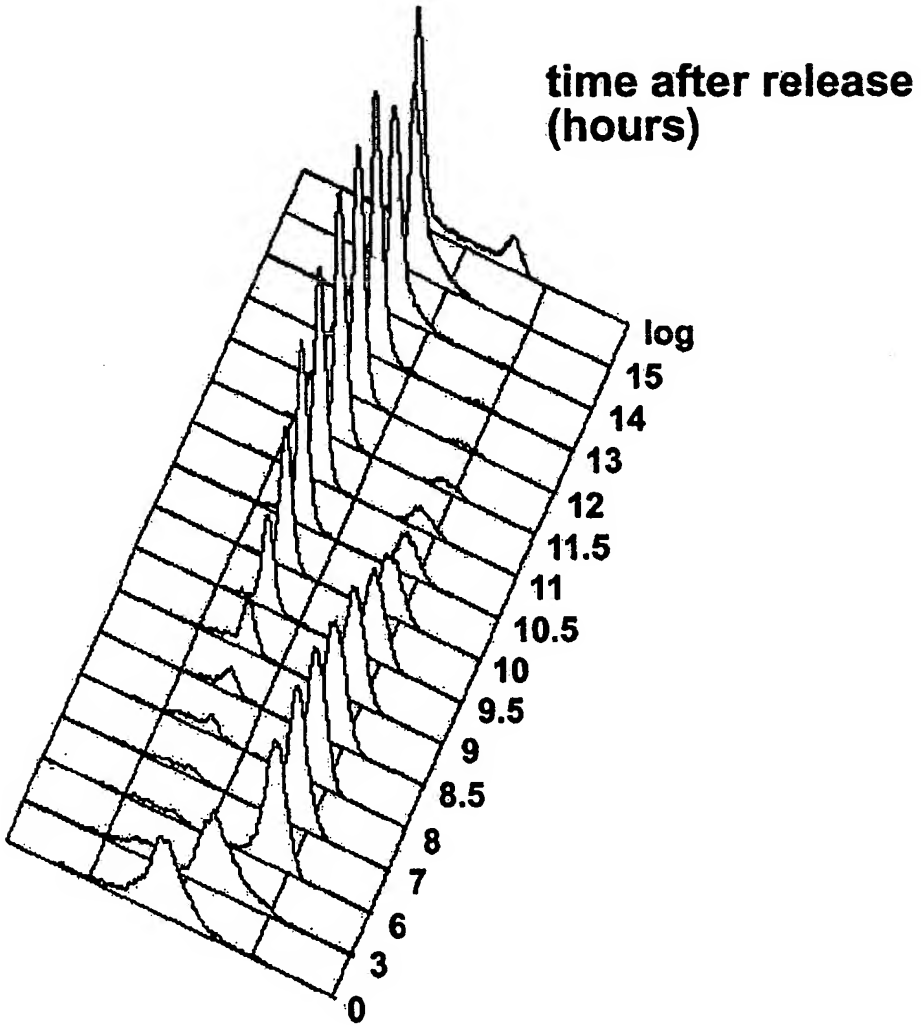
time after release
(hours)
log
15
14
13
12
11.5
11
10.5
10
9.5
9
8.5
8
7
6
3
0

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Fig. 11 b

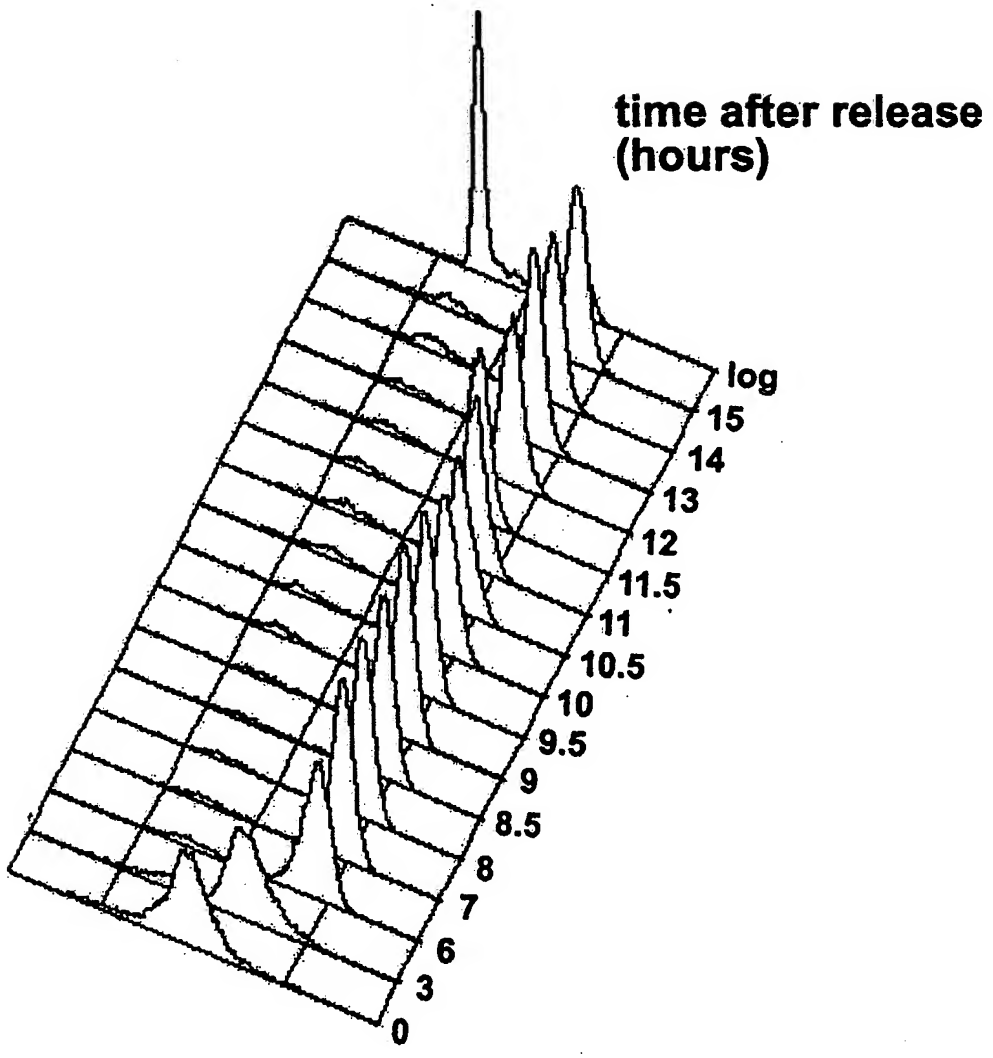
time after release
(hours)
log
15
14
13
12
11.5
11
10.5
10
9.5
9
8.5
8
7
6
3
0

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Fig. 11 c

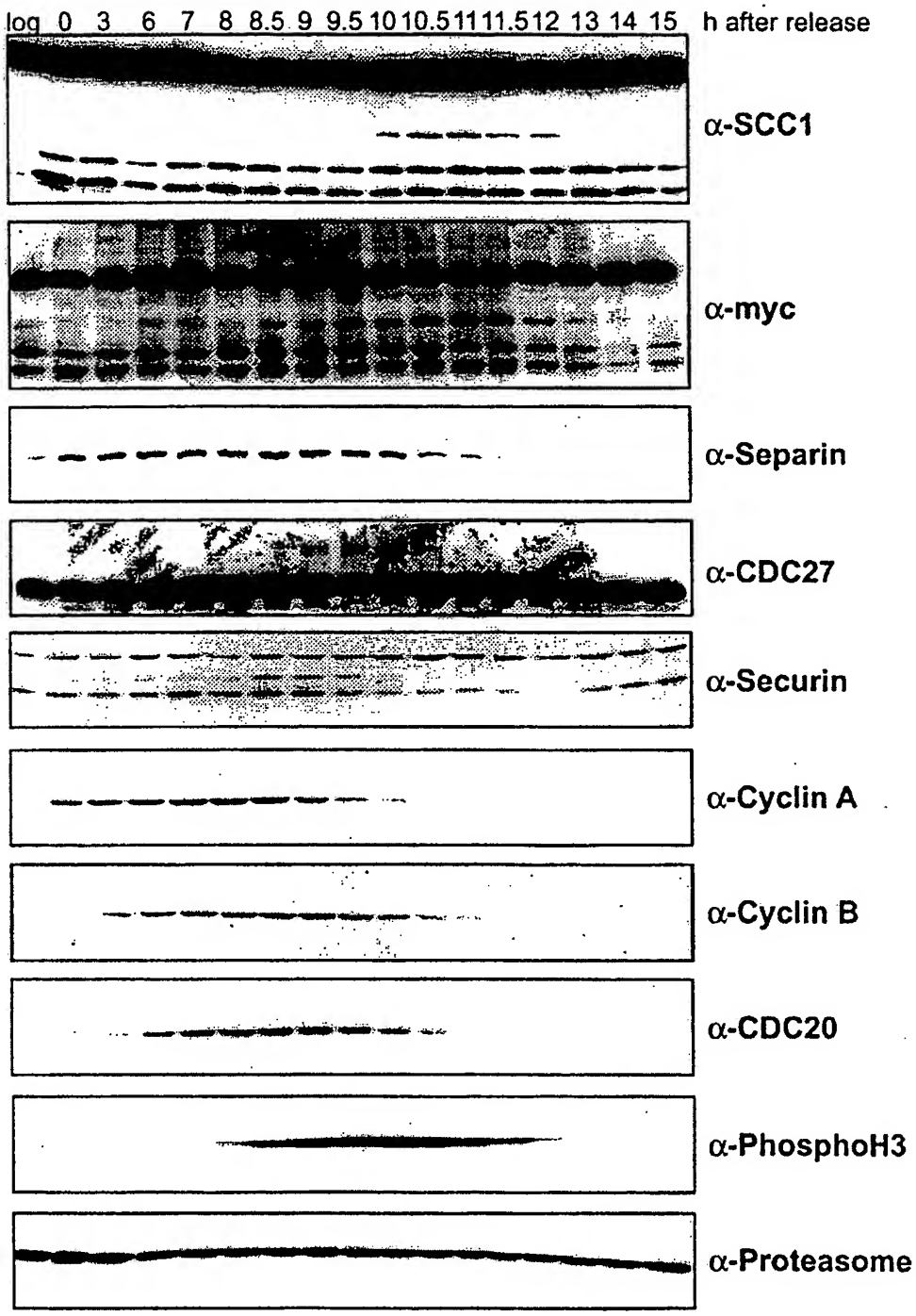
log 0 3 6 7 8 8.5 9 9.5 10 10.5 11 11.5 12 13 14 15 h after release
α-SCC1
α-myc
α-Separin
α-CDC27
α-Securin
α-Cyclin A
α-Cyclin B
α-CDC20
α-PhosphoH3
α-Proteasome

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Fig. 11 d

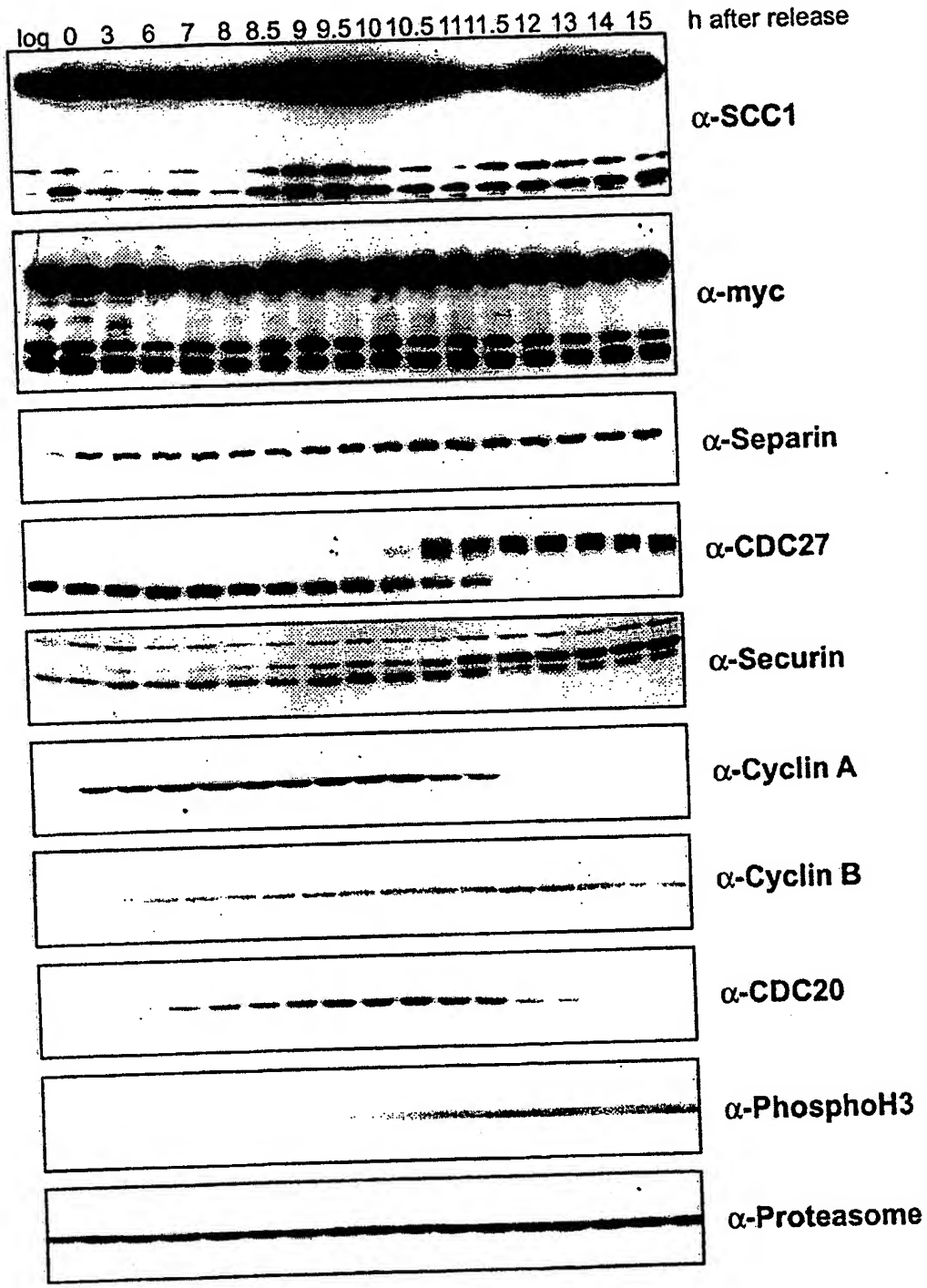
log 0 3 6 7 8 8.5 9 9.5 10 10.5 11 11.5 12 13 14 15
h after release
α-SCC1
α-myc
α-Separin
α-CDC27
α-Securin
α-Cyclin A
α-Cyclin B
α-CDC20
α-PhosphoH3
α-Proteasome

Fig. 12 a

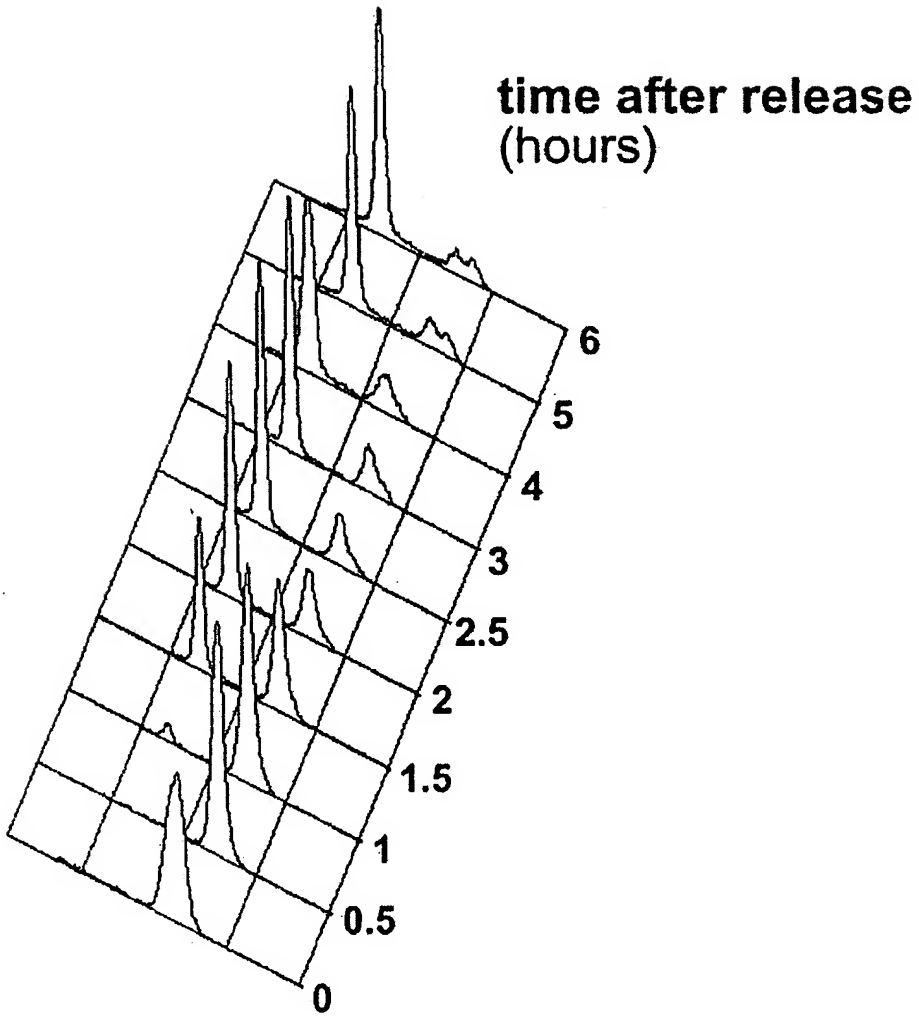

Fig. 12 b

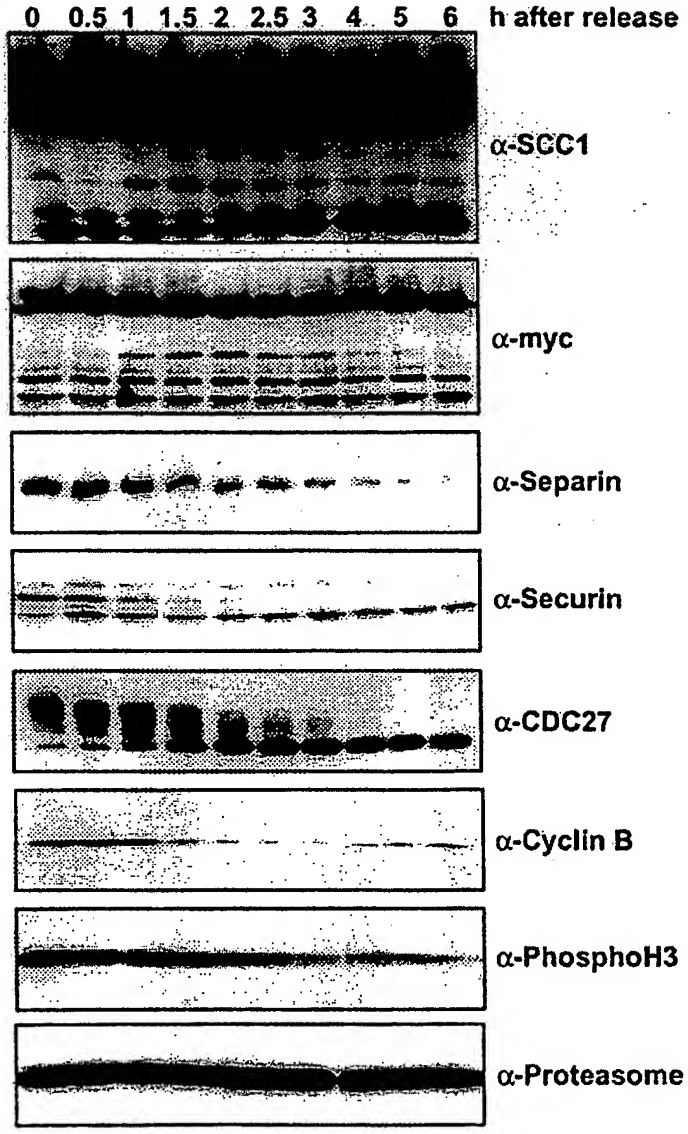
0 0.5 1 1.5 2 2.5 3 4 5 6 h after release
α-SCC1
α-myc
α-Separin
α-Securin
α-CDC27
α-Cyclin B
α-PhosphoH3
α-Proteasome

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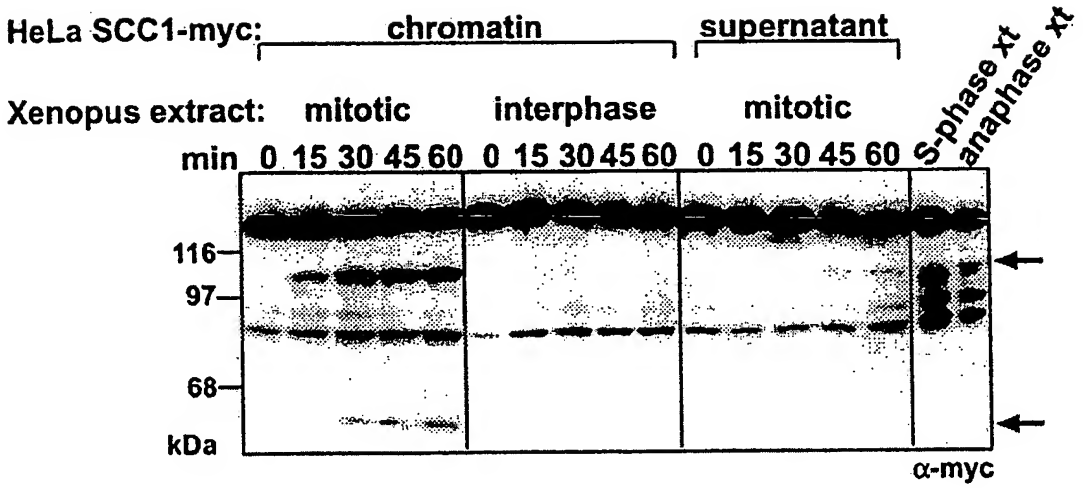
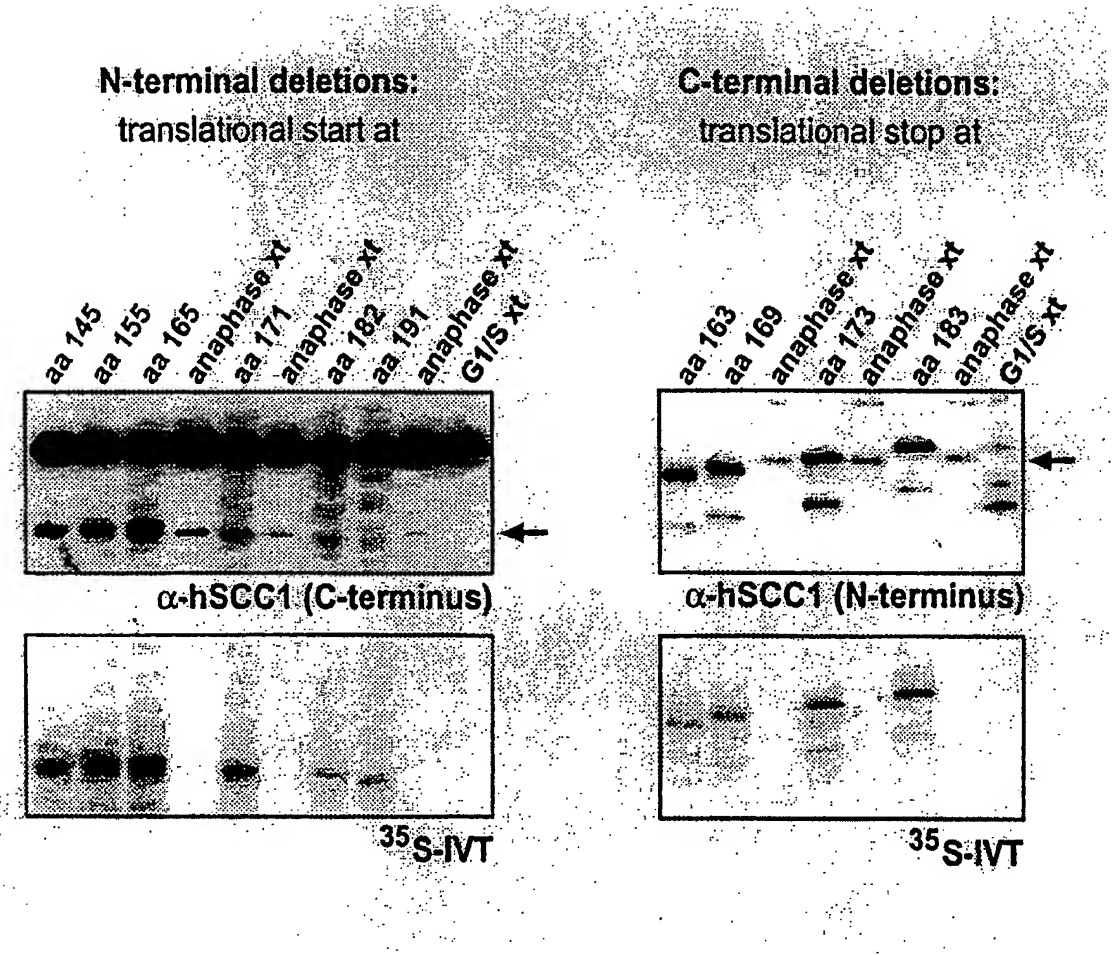
Fig. 13


HeLa SCC1-myc:
chromatin
supernatant
Xenopus extract:
mitotic
interphase
mitotic
S-phase xt
anaphase xt
min 0 15 30 45 60 0 15 30 45 60 0 15 30 45 60
116
97
68
kDa
α-myc

24/24
Fig. 14


N-terminal deletions:
translational start at
C-terminal deletions:
translational stop at
aa 145
aa 155
aa 165
anaphase xt
aa 171
anaphase xt
aa 182
aa 191
anaphase xt
G1/S xt
aa 163
aa 169
anaphase xt
aa 173
anaphase xt
aa 183
anaphase xt
G1/S xt
α-hSCC1 (C-terminus)
α-hSCC1 (N-terminus)
35S-IVT
35S-IVT

SEQUENCE LISTING

<110> Boehringer Ingelheim International GmbH

<120> Compounds modulating sister chromatid separation and methods for identifying same

<130> 14047peptide

<140>
<141>

<160> 1

<170> PatentIn Ver. 2.1

<210> 1
<211> 18
<212> PRT
<213> Homo sapiens

<400> 1

Met Asp Asp Arg Glu Ile Met Arg Glu Gly Ser Ala Phe Glu Asp Asp
1 5 10 15

Asp Met

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/EP 00/01183

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/55 A01H5/00 C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A01H G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 15749 A (PROTOTEK INC) 15 June 1995 (1995-06-15) page 1 -page 3, line 21 page 6, line 23 - line 26	10-12,15
X	WO 98 49190 A (CORTECH INC ;LEIMER AXEL H (US); SPRUCE LYLE W (US); CHERONIS JOHN) 5 November 1998 (1998-11-05) page 1 -page 2 page 25, line 19 - line 27	10-12,15

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 June 2000	29/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Fernandez y Branas,F

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CIOSK R ET AL: "An ESP1 /PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast." CELL, (1998 JUN 12) 93 (6) 1067-76., XP002118931 cited in the application the whole document	1-9
A	WO 98 27994 A (UNIV TEXAS) 2 July 1998 (1998-07-02) the whole document	1-3
A	CLARK D A ET AL: "PROTEASE INHIBITORS SUPPRESS IN VITRO GROWTH OF HUMAN SMALL CELL LUNG CANCER" PEPTIDES, vol. 14, no. 5, 1993, pages 1021-1028, XP002914836 the whole document	1,2
A	DATABASE EMBL 'Online! AC D79987, 1995 NOMURA N ET AL: "Prediction of the coding sequences of unidentified human genes. V. The coding sequences of 40 new genes (KIAA 0161-KIAA0200) deduced by analysis of the cDNA clones from human cell line KG1" XP002139909 abstract & DNA RES. Vol 3, 17-24, 1996	1-16
T	UHLMANN F. ET AL: "Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1" NATURE, vol. 400, July 1999 (1999-07), pages 37-42, XP002118932 the whole document	1-3

4

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 10-16 (partially)

Present claims 10-16 relate to a compound defined by reference to a desirable characteristic or property, namely the inhibition of "proteases with separin-like activity".

The claims cover all compounds having this characteristic or property, whereas the application provides no support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for the general inventive idea of the inhibition the so called "separin-like protease" for the uses defined in claims 10-15.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ...nal Application No
PCT/EP 00/01183

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9515749 A	15-06-1995	US 5486623 A	23-01-1996
		AU 1266495 A	27-06-1995
		CA 2177495 A	15-06-1995
		EP 0731696 A	18-09-1996
		JP 9506368 T	24-06-1997
		US 5714484 A	03-02-1998
		US 5663380 A	02-09-1997
		US 5925772 A	20-07-1999
WO 9849190 A	05-11-1998	US 6004933 A	21-12-1999
		AU 7155698 A	24-11-1998
		EP 0979242 A	16-02-2000
		AU 3965199 A	08-11-1999
		WO 9954317 A	28-10-1999
WO 9827994 A	02-07-1998	AU 5384898 A	17-07-1998
		EP 0956032 A	17-11-1999

Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1

Frank Uhlmann, Friedrich Lottspeich* & Kim Nasmyth

Research Institute of Molecular Pathology, A-1030 Vienna, Austria
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Cohesion between sister chromatids is established during DNA replication and depends on a multiprotein complex called cohesin. Attachment of sister kinetochores to the mitotic spindle during mitosis generates forces that would immediately split sister chromatids were it not opposed by cohesion. Cohesion is essential for the alignment of chromosomes in metaphase but must be abolished for sister separation to start during anaphase. In the budding yeast *Saccharomyces cerevisiae*, loss of sister-chromatid cohesion depends on a separating protein (separin) called Esp1 and is accompanied by dissociation from the chromosomes of the cohesion subunit Scc1. Here we show that Esp1 causes the dissociation of Scc1 from chromosomes by stimulating its cleavage by proteolysis. A mutant Scc1 is described that is resistant to Esp1-dependent cleavage and which blocks both sister-chromatid separation and the dissociation of Scc1 from chromosomes. The evolutionary conservation of separins indicates that the proteolytic cleavage of cohesion proteins might be a general mechanism for triggering anaphase.

The separation of sister chromatids at the metaphase-to-anaphase transition is one of the most dramatic events of the eukaryotic cell cycle. As cells enter mitosis, chromosome condensation during prometaphase resolves the bulk of each chromatid's chromatin from that of its sister[1,2]. Chromatids nevertheless remain paired along their entire length during the attachment of chromosomes to the mitotic spindle. Cohesion between sisters resists the pulling forces exerted by microtubles attached to sister kinetochores[3] and thereby ensures that sister chromatids attach to microtubules emanating from opposite spindle poles[4,5]. It has long been suspected that destruction of sister-chromatid cohesion, rather than a major change in traction exerted by the spindle, is responsible for the sudden separation of sister chromatids at the metaphase-to-anaphase transition[3,4]. It is not known what triggers this event in the eukaryotic cell cycle.

There are important clues as to the molecular nature of the cohesive structures that holds sisters together and the mechanism by which it is suddenly broken at the onset of anaphase[6]. In *S. cerevisiae*, cohesion between sister chromatids depends on a multisubunit complex, called cohesin, which contains at least four subunits: Scc1, Scc3, Smc1 and Smc3 (refs 7–9). Cohesion is established during DNA replication with the help of Scc2 and Eco1 (refs 9–11). A similar cohesin complex has been implicated in sister-chromatid cohesion in *Xenopus* extracts[2].

In yeast, there is a sudden change in the state of cohesin at the metaphase-to-anaphase transition: two cohesin subunits, Scc1 and Scc3, suddenly disappear from chromosomes at the point when sister chromatids separate[7,9]. The dissociation of Scc1 from chromosomes and the separation of sister chromatids both depend on a 'separin' protein called Esp1 (ref. 12). The existence of Esp1 homologues in many eukaryotes, including humans, suggests that separins have a fundamental and conserved role in chromosome segregation[13–15].

For much of the cell cycle, Esp1 is tightly bound by the anaphase inhibitor Pds1 (ref. 12), whose destruction at the metaphase-to-anaphase transition is triggered by ubiquitination due to the anaphase-promoting complex (APC)[16]. The APC requires an activator protein, Cdc20, to mediate Pds1 destruction[17]. In *S. cerevisiae*, the only role of the APC in promoting sister separation is to destroy Pds1 (refs 12, 18). We now investigate the mechanism by which Esp1 dissolves sister-chromatid cohesion once it has been liberated from Pds1.

Esp1 controls Scc1 chromosome association

The displacement of Scc1 from chromosomes at the metaphase-to-anaphase transition might be a direct effect of Esp1 activity. Alternatively, it might just be a consequence of sister-chromatid separation initiated by Esp1. We therefore examined the mechanism that prevents the association of Scc1 with chromosomes during early G1 phase. Scc1 is destroyed during anaphase and is normally not resynthesized until late G1 in the next cell cycle[7]. However, even when Scc1 is synthesized in early-G1 cells from the galactose-inducible *GAL1-10* promoter, it fails to bind stably to chromosomes[10]. We repeated this experiment using cells arrested in a G1-like state with the mating pheromone α-factor (Fig. 1a). Scc1, induced during pheromone arrest, accumulated in the nuclei of most cells, but bound to chromosomes only weakly, if at all. Furthermore, the protein rapidly disappeared from cells after expression was shut off by addition of glucose (Fig. 1b). When the experiment was repeated with *esp1-1* mutant cells, Scc1 bound to chromosomes with high efficiency and remained associated even after synthesis was terminated (Fig. 1b). This result indicates that Esp1 prevents the stable association of Scc1 with chromosomes during G1 phase, in addition to causing dissociation of Scc1 from chromosomes when sister chromatids separate. Esp1 may therefore have a direct role in removing Scc1 from chromosomes.

An *in vitro* Scc1-dissociation assay

To investigate the mechanism by which Esp1 causes Scc1 to dissociate from chromosomes, we assayed this process *in vitro* (Fig. 2). A crude preparation of yeast chromatin[19], isolated from cells arrested in a metaphase-like state by nocodazole, was incubated with soluble extracts from *esp1-1* mutant cells that either had or had not been induced to overexpress wild-type Esp1 from the *GAL1-10*

promoter. After incubation with both types of extract, the chromatin fraction was again separated from the supernatant by centrifugation and the levels of haemagglutinin(HA)-epitope-tagged Scc1 in chromatin and supernatant fractions were analysed by SDS–PAGE and subsequent immunoblotting. About 70% of the total Scc1 in nocodazole-blocked cells is tightly associated with chromatin[9] and is therefore present in the starting chromatin fraction, which served as substrate. Most Scc1 remained in the chromatin fraction following incubation with extract prepared from *esp1-1* mutant cells, but almost all disappeared from the chromatin fraction after incubation with extract containing overexpressed Esp1 (Fig. 2). Surprisingly, Scc1 induced to dissociate from chromatin by Esp1 appeared in the supernatant fraction as a cleaved product (Fig. 2). Both dissociation of Scc1 from chromatin and its cleavage were inhibited by Pds1 translated in reticulocyte lysate, but not by a control lysate (Fig. 2). We also detected a small amount of Scc1-cleavage activity in extracts prepared from cells not overexpressing Esp1 if they were arrested in G1, and in extracts from cycling cells lacking Pds1 (data not shown). Esp1 also caused ~50% of the Scc3 cohesin subunit[9] to dissociate from chromatin without cleavage. The association of Smc proteins and histone H2B1 with chromatin was unaffected by Esp1-containing extracts (data not shown).

We next investigated the requirements for Scc1 dissociation *in vitro*. It has been suggested that a transient calcium wave in mitotic cells might trigger sister-chromatid separation[20], but Scc1 cleavage was unaltered by addition of the calcium chelator EGTA or by an excess of free calcium. The reaction was also not inhibited by inhibitors of kinases or phosphatases (data not shown). This suggests that the dissociation of Scc1 from metaphase chromatin *in vitro* is neither induced by calcium nor by *de novo* phosphorylation/dephosphorylation. ATP depletion of extracts or addition of the proteasome inhibitor LLNL did not prevent Scc1 cleavage (data not shown), indicating that Scc1 cleavage is probably not due to APC-mediated ubiquitination. We attempted to characterize the proteolytic activity by using protease inhibitors from the four known classes, and found that Scc1 cleavage was only inhibited by high concentrations (10 mM) of *N*-ethylmaleimide.

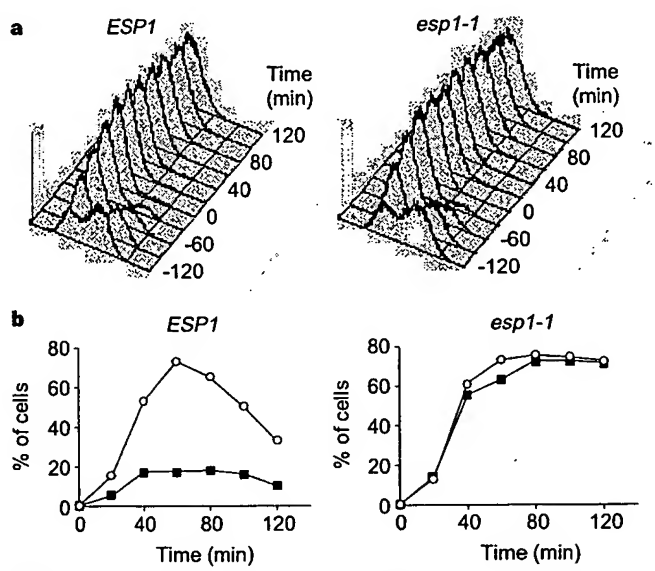


Figure 1 Esp1-dependent chromosome association of Scc1 in G1. **a**, Strains K7466 (*MATa ESP1 SCC1 GAL-SCC1myc18*) and K7468 (*MATa esp1-1 SCC1 GAL-SCC1myc18*) were arrested in G1 with α-factor for 120 min (time point zero). FACScan analysis showed that cells stayed arrested during the experiment. **b**, Scc1-Myc18 was induced for 60 min, then cells were transferred to medium containing glucose to repress Scc1-Myc18. Expression of Scc1-Myc18 was seen by whole-cell *in situ* staining (circles), and chromosome binding of Scc1-Myc18 was observed by using chromosome spreads (squares).

Scc1 cleaved at anaphase onset *in vivo*

To test whether Esp1-induced cleavage of Scc1 also occurs *in vivo* at the onset of anaphase, we used a yeast strain in which expression of the APC-activator Cdc20 is under the control of the galactose-inducible *GAL1-10* promoter[21]. Cells from this strain were arrested in metaphase by incubation in galactose-free medium and then induced to undergo synchronous anaphase by addition of galactose. Sister-chromatid separation, visualized by the binding to *tet* operators close to CenV of *tet* repressor tagged with green fluorescent protein (GFP) (ref. 7), occurred in most cells within 15 min of Cdc20 induction, and Scc1 dissociated from chromosomes at a similar rate (Fig. 3a, b). We detected a small amount of Scc1 cleavage product of the same size as that seen *in vitro* in cycling cells, but not in cells arrested in metaphase. The cleavage product appeared 15 min after release into anaphase, simultaneously with sister-chromatid separation and the dissociation of Scc1 from chromosomes (Fig. 3c). Full-length Scc1 remaining in cells at this time might originate from the soluble pool of Scc1, whose cleavage is unnecessary for sister separation. Soluble Scc1 in the supernatant fraction of chromatin preparations makes a poor substrate in our *in vitro* cleavage assay (data not shown).

To establish whether cleavage of Scc1 during anaphase depends on Esp1, we compared wild-type and *esp1-1* mutant cells after their release from *GAL–CDC20* arrest at 35 °C. The extent of sister-chromatid separation, Scc1 dissociation from chromosomes (data not shown), and Scc1 cleavage (Fig. 3d) was greatly reduced in the *esp1-1* mutant. We conclude that Esp1 promotes cleavage of Scc1 and its dissociation from chromosomes both *in vivo* and *in vitro*.

A cleavage-resistant Scc1

To determine whether Esp1-mediated cleavage of Scc1 is a cause or a consequence of its dissociation from chromosomes, we first identified the Scc1-cleavage site, with a view to producing a cleavage-resistant mutant. The C-terminal Scc1-cleavage product in anaphase cells (Fig. 3) was immunoprecipitated from cell extracts. Amino-terminal sequence analysis of the fragment showed that cleavage had occurred between a pair of arginine

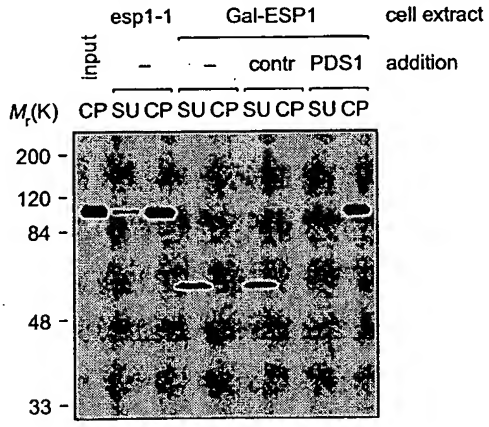


Figure 2 *In vitro* assay for Scc1 dissociation from chromatin. Chromatin was prepared as described[19] from strain K7563 (*MATα SCC1-HA6*) arrested in metaphase by nocodazole treatment. Proteins in the chromatin preparation were resolved by SDS-PAGE; Scc1-HA6 was detected by western blotting (input). This chromatin preparation was resuspended in the indicated extracts, with or without addition of 50% (v/v) *in vitro* translation reactions, as indicated. After incubation, aliquots of the supernatant fraction (SU) and the chromatin fraction (CP) of each reaction were analysed.

residues at positions 268 and 269. The first of these arginine residues was then mutated to aspartic acid (R268D), tagged at the C terminus with HA epitopes, and expressed from the *GAL1-10* promoter: expression of the mutant protein had little effect on cell proliferation. To test whether the R268D mutation had abolished cleavage, we used chromatin from cells expressing it as a substrate in the Esp1 assay. We found that there was no cleavage at site 268, but that the mutant protein was still cleaved in an Esp1-dependent manner (Fig. 4a). The C-terminal cleavage product was now about 10K larger. To identify the second cleavage site, we looked for sequences in Scc1 that are similar to those around the C-terminal cleavage site and found a 5-out-of-7 amino-acid match at position 180 (Fig. 4b). We mutated the arginine before this putative cleavage site to aspartate (R180D).

We next compared the effect of expressing wild-type Scc1, R180D and R268D single-mutant proteins, and the R180D/R268D double-mutant protein from the *GAL1-10* promoter. Neither the single-mutant proteins nor the wild-type protein greatly affected cell proliferation, but expression of the double-mutant protein was lethal (data not shown). We used chromatin from cells transiently expressing the double-mutant protein in our Esp1 assay and found that the R180D/R268D double-mutant protein (Scc1RR-DD) was no longer cleaved. Furthermore, it failed to dissociate from chromosomes (Fig. 4a). The small amount of 'leakage' of Scc1 from chromatin into the supernatant was Esp1-independent (data not shown).

To obtain the N- and C-terminal cleavage products simultaneously, we used as a substrate chromatin from a strain expressing Scc1 tagged N-terminally with a Myc epitope and C-terminally with HA epitope (Fig. 4b). Esp1-mediated cleavage produced a single HA-tagged cleavage fragment but two Myc-tagged fragments, the smaller of which was more abundant (Fig. 4c). These results indicate that all molecules were cleaved at the C-terminal site but not all of them were cleaved at the N-terminal site. A similar pattern of N-terminal cleavage fragments was obtained during anaphase *in vivo* (data not shown).

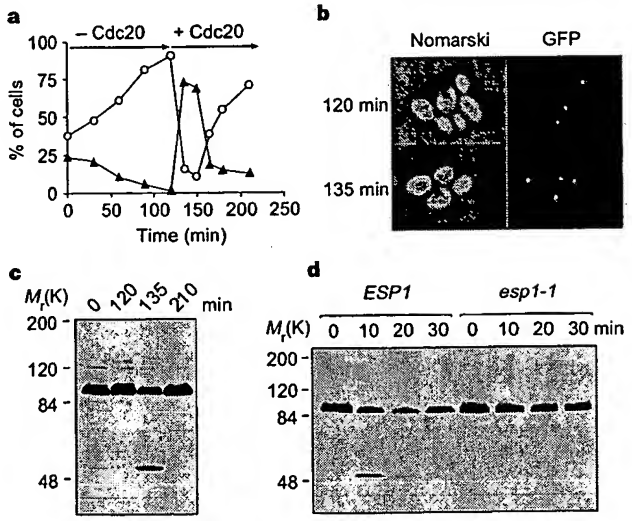


Figure 3 Scc1p cleavage at anaphase onset *in vivo*. **a**, Metaphase arrest and release using Cdc20 depletion of strain K7677 (*MATα cdc20Δ GAL-CDC20 SCC1-HA3 TetR-GFP TetOs*). Scc1-HA3 bound to chromosomes (circles), and the fraction of cells containing two separated GFP dots (triangles). **b**, Examples of cells in the arrest at 120 min, and 15 min after release. **c**, Western blot analysis of Scc1-HA3 in whole-cell extracts prepared from cells at the indicated time points. **d**, As **c**, except that strain K7677 and K8054 (*MATa esp1-1 cdc20Δ GAL-CDC20 SCC1-HA3 TetR-GFP TetOs*) were arrested and released at 35 °C.

Non-cleavable Scc1 and sister separation

To investigate why cells expressing the non-cleavable Scc1RR-DD double mutant cannot proliferate, we used centrifugal elutriation to isolate G1 cells from a culture growing without expression of Scc1RR-DD, which were then incubated in the presence and absence of galactose to induce Scc1RR-DD from the *GAL1-10* promoter (Fig. 5). To minimize the duration of mutant protein expression, cells grown in the presence of galactose were transferred to glucose-containing medium after 135 min, when most cells had replicated their DNA (Fig. 5a). In the absence of galactose, sister separation and the dissociation from chromosomes of endogenous Myc-tagged Scc1 occurred simultaneously about 60 min after DNA replication (Fig. 5b–d). Transient expression of Scc1RR-DD, tagged with HA epitope, almost completely prevented sister-chromatid separation (Fig. 5b) but did not affect binding and dissociation of endogenous wild-type Scc1 (Fig. 5c). The mutant protein remained tightly associated with chromosomes long after the endogenous wild-type protein had disappeared (Fig. 5c, d). Scc1RR-DD bound to chromosomes immediately following induction in G1, when wild-type Scc1 is prevented from binding by Esp1 (compare to Fig. 1 and

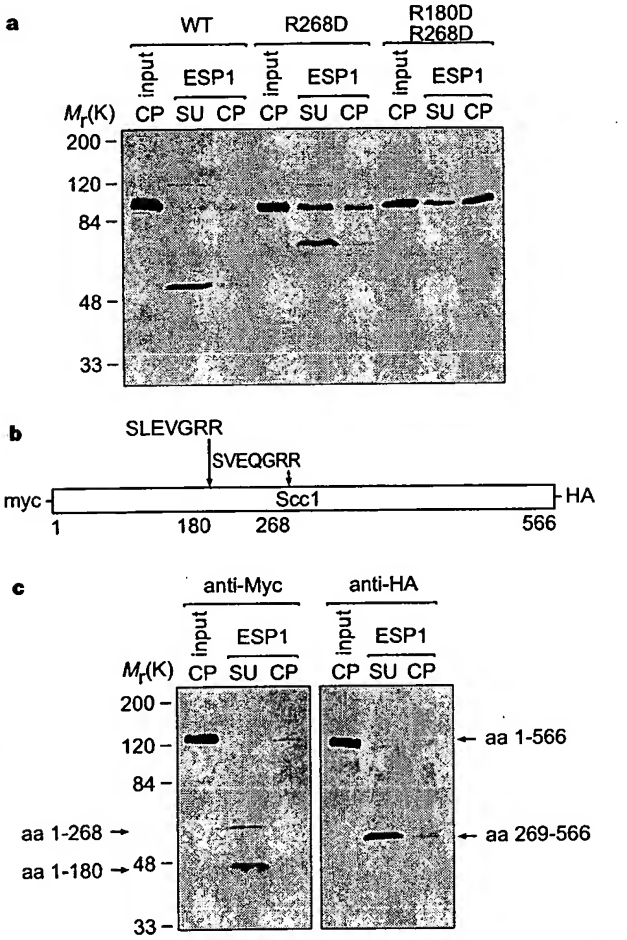


Figure 4 Characterization of the Scc1 cleavage sites. **a**, Strains K8097 (*MATa SCC1-myc18 GAL-SCC1-HA3 TetR-GFP TetOs*), K8099 (*MATa SCC1-myc18 GAL-SCC1(R268D)-HA3 TetR-GFP TetOs*) and K8101 (*MATa SCC1-myc18 GAL-SCC1(R180D, R268D)-HA3 TetR-GFP TetOs*) were grown in medium containing 2% raffinose. Expression of the respective Scc1 variant was induced for 4 h, and cells were arrested by nocodazole treatment. Chromatin was prepared and used in the Esp1 assay. The 120K bands are HA-cross-reacting proteins. WT, wild type. **b**, The cleavage sites in Scc1. **c**, Chromatin was prepared from strain K7768 (*MATα myc9-SCC1-HA6*). Scc1 (ref. 8) and the derived cleavage fragments (aa, amino-acid residues) migrate abnormally slowly during SDS-PAGE, as shown by western blotting with the indicated antibodies.

ref. 10). The failure of Scc1RR-DD to dissociate from chromosomes was not an artefact due to transient overexpression of the protein from the *GAL1-10* promoter, because wild-type Scc1 expressed similarly dissociates from chromosomes with normal kinetics[10].

Expression of the Scc1RR-DD mutant protein caused a transient delay of cytokinesis for 20 min (Fig. 5b), after which cells divided without having separated sister chromatids. Progeny with abnormal DNA contents were produced (Fig. 5a), resembling *esp1-1* mutant cells incubated at the restrictive temperature[22]. The dissociation from chromosomes of wild-type protein on cue shows that Esp1 activity was not impaired in these cells. The failure of sister chromatids to separate even when Esp1 was active also prevented elongation of the mitotic spindle (data not shown), consistent with the idea that loss of cohesion triggers anaphase.

To determine the phenotype of single- and double-mutant Scc1 proteins when expressed from the natural *SCC1* promoter, we transferred the mutations to *SCC1* carried on a centromeric vector. Plasmids carrying the wild-type or either of the single-mutant genes transformed wild-type and *scc1-73* strains and complemented the temperature-sensitive phenotype of the *scc1–73* mutation. No transformants expressing the R180D/R268D double mutant were obtained (data not shown). Together, our results indicate that cleavage of Scc1 at one of two sites is necessary both for sister-chromatid separation and for the dissociation of Scc1 from chromosomes.

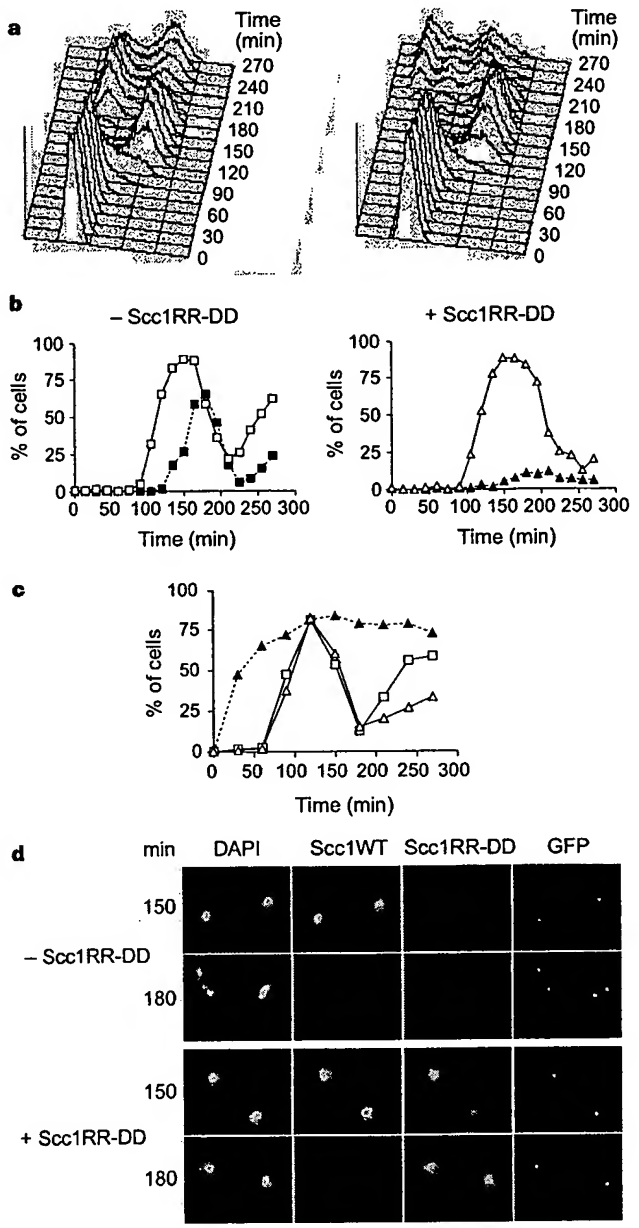


Figure 5 Expression of non-cleavable Scc1 prevents sister chromatid separation. **a**, DNA content as unbudded G1 cells of strain K8101 were released into the cell cycle with or without induction of the Scc1RR-DD mutant. **b**, Budding index (without Scc1RR-DD, open squares; with Scc1RR-DD, open triangles) and percentage of cells with separated sister chromatids (filled symbols). **c**, Scc1 chromosome association. Endogenous wild-type Scc1-Myc18 in cells without (squares) and with Scc1RR-DD expression (open triangles). Scc1RR-DD was tagged with HA epitopes (filled triangles). **d**, Examples of chromosome spreads at 150 min in metaphase and at 180 min when most cells of the control culture had undergone anaphase. DNA was stained with DAPI, Scc1-Myc18 was detected with rabbit anti-Myc antiserum and Cy5-conjugated secondary antibody, Scc1RR-DD-HA3 was detected with mouse monoclonal antibody 16B12 and Cy3-conjugated secondary antibody. Sister chromatids of chromosome V were visible by the GFP dots.

Non-cleavable Scc1RR-DD is functional

To test whether Scc1RR-DD is fully functional apart from its non-cleavability, we first checked whether the double-mutant protein could establish cohesion by itself between sister chromatids in the absence of endogenous Scc1 function, and then whether cohesion established by Scc1RR-DD was dependent on other cohesin subunits. We used centrifugal elutriation to isolate G1 cells of *scc1–73* and *smc3–42* mutant strains[7] that could express Scc1RR-DD from the *GAL1-10* promoter, then incubated them in the presence or absence of Scc1RR-DD at 35 °C, a restrictive temperature for both mutations. In the absence of galactose, sister chromatids separated prematurely in both mutants and failed to segregate to opposite poles of the cell[7] (Fig. 6). Expression of Scc1RR-DD suppressed premature sister-chromatid separation in *scc1-73* mutant cells but not in *smc3-42* cells (Fig. 6). Thus, Scc1RR-DD alone fulfils the cohesion function of Scc1. The cohesion due to Scc1RR-DD depends on Smc3, as does that produced by wild-type Scc1,

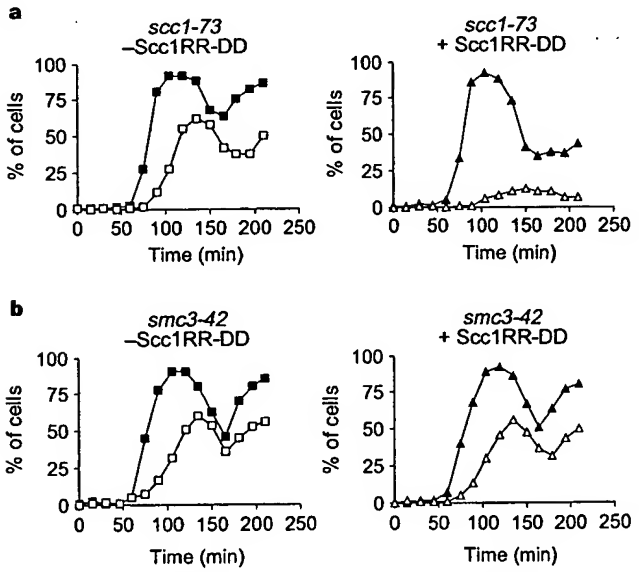


Figure 6 Scc1RR-DD is a functional Scc1 variant. **a**, G1 cells of strain K8103 (*MATa scc1-73 GAL-SCC1(R180D,R268D)-HA3 TetR-GFP TetOs*) were released at 35 °C into medium containing or lacking galactose. The budding index (filled symbols) and the percentage of cells containing separated sister chromatids (open symbols) are shown. **b**, As **a**, except that strain K8149 (*MATa smc3-42 SCC1-myc18 GAL-SCC1(R180D,R268D)*-HA3 TetR-GFP TetOs) was used.

suggesting that Scc1RR-DD differs from wild-type only in its susceptibility to cleavage by Esp1.

Cohesin cleavage triggers anaphase

Our results indicate how cohesion between sister chromatids might be destroyed at the onset of anaphase in yeast (Fig. 7a). As Scc1 is required to maintain cohesion until the metaphase-to-anaphase transition[7–9], its disappearance from chromosomes should destroy cohesion. We have shown that cleavage of Scc1 mediated by the separin Esp1 at the exact point when sister chromatids separate is necessary for sister-chromatid separation, for dissociation of Scc1 from chromosomes, and for the movement of spindle poles to opposite ends of the cell. We propose that sister separation in yeast is triggered by the sudden cleavage of Scc1, although what brings this about is not fully understood. Proteolysis of Pds1 shortly before the metaphase-to-anaphase transition is important: Pds1 inhibits Esp1-dependent cleavage *in vitro* and is destroyed suddenly by APC^{Cdc20} *in vivo* so that sister chromatids can separate[16,17]. The inhibition of APC^{Cdc20} by Mad2 that occurs when chromosomes fail to attach correctly to the mitotic spindle[18,23,24] prevents the destruction of Pds1 and so blocks the onset of anaphase (hence the dubbing of Pds1 and homologues like Cut2 (ref. 25) as securins). Although destruction of Pds1 is necessary for sister separation, it is not sufficient. The disappearance of Scc1 from chromosomes is tightly regulated in yeast cells lacking Pds1 (ref. 26), so a second pathway must exist that regulates either Esp1 activity or the susceptibility of Scc1 to Esp1-dependent cleavage.

Given that Smc1 and Smc3 may form an antiparallel heterodimer[27] and that the link between sisters is a symmetrical, we suggest that Scc1 and Scc3 hold the two Smc1/3 heterodimers together, with each being bound to sister DNA molecules[28] (Fig. 7a); separin could then cleave sister chromatids apart. Cleavage also destabilizes the association of Scc1 with the rest of the cohesin complex. Our results do not indicate whether it is cleavage of Scc1, its subsequent dissociation from the cohesin complex, or both combined that triggers sister-chromatid separation.

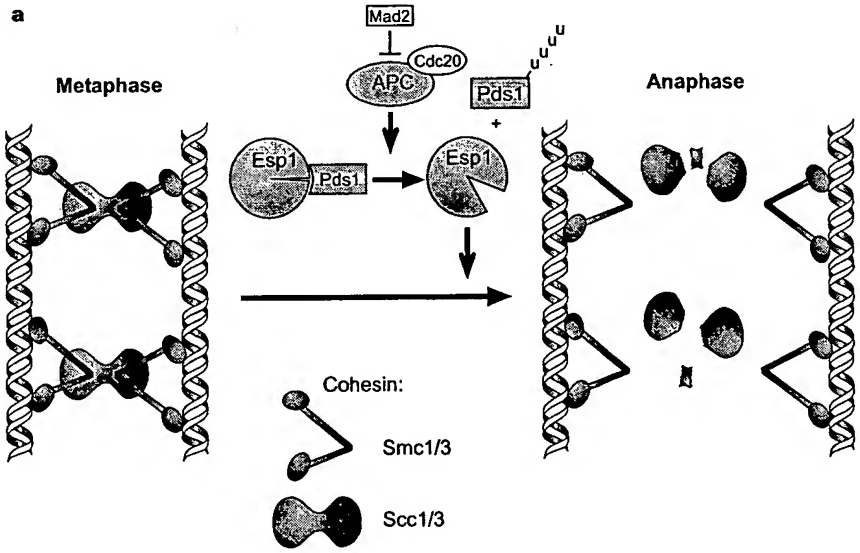
Is cohesin cleavage general?

To investigate whether Scc1 was separin's only target, we searched for yeast proteins containing the Scc1 cleavage-site consensus sequences SxExGRR. Only one protein gave a convincing match: we call this protein Rec8 (ref. 29) on the basis of its homology to the *rec8* gene product of fission yeast (Fig. 7b). Rec8 is a member of the Scc1 family and contains two SxExGRK motifs; it is only expressed in meiotic cells and, unlike Scc1, is essential for sister-chromatid cohesion during meiosis I and II (ref. 30; and F. Klein, S. Buonomo and K.N., unpublished results). Rec8 seems to replace Scc1 during meiosis, and cleavage of Rec8 might be crucial for separating sister chromatids during meiosis.

We have not been able to detect Scc1 cleavage motifs in homologous proteins from animals, but the equivalent protein in fission yeast, Rad21 (ref. 31), does contain two near matches in a similar region of the protein to those from Scc1 (Fig. 7b). Most cohesin in animal cells dissociates from chromosomes during prometaphase[2]. A key question for the future is therefore whether the target for animal cell separins is a small fraction of the cohesin pool that persists on metaphase chromosomes or some other cohesion protein.

Is the Esp1 separin a protease?

Although we have not directly determined the identity of the protease that cleaves Scc1, our finding that the cleavage activity in extracts is roughly proportional to their Esp1 concentration (data not shown) raises the possibility that Esp1 is itself the protease. Esp1 and its homologues in other organisms are all large proteins of



b

ScScc1	174	TSLEVGRRF	182
ScScc1	262	NSVEQGRRL	270
ScRec8	425	SSVERGRKR	433
ScRec8	447	RSHEYGRKS	455
SpRad21	173	LSIEAGRNA	181
SpRad21	225	ISIEVGRDA	233
SpRec8	378	SEVEVGRDV	386

Figure 7 Model for separin action on cohesin, and conservation of potential cohesin cleavage sites. **a**, Proteolytic cleavage of one of cohesin's subunits is necessary for sister separation, suggesting that cohesin complexes link sister chromatids. Mad2 is included in the scheme as a known inhibitor of APC^{Cdc20} (ref. 24). **b**, Alignment of known and putative cohesin cleavage sites. Sequence motifs similar to the consensus between the two Scc1 cleavage sites were searched for using the HMMER algorithm[38].

relative molecular mass ~200K (refs 13, 15, 22, 32). Most of their sequences are not highly conserved, but they all contain a conserved C-terminal domain, which might be responsible for a proteolytic activity. This 'separin domain' does not resemble any known protease. However, the insensitivity of the cleavage reaction to many known protease inhibitors suggests that the protease may have a novel mechanism of action. If Esp1 is not itself the protease, then it might instead be an allosteric effector of a protease, which either resides on chromatin or is present in the soluble fraction. Indeed, Esp1 might have functions in addition to Scc1 cleavage[37]. It will be necessary to purify Esp1 and provide it with a more clearly defined substrate to answer these questions. □

Methods

Plasmids and strains. The Scc1 coding sequence was cloned under control of the *GAL1-10* promoter into a YIplac128 derived vector[33]. A DNA fragment encoding 3 tandem HA epitopes was inserted into a *Not*I restriction site introduced by PCR at the C terminus of *SCC1*. Site-directed mutagenesis was performed by exchanging restriction fragments from Scc1 with PCR fragments obtained using primers containing the desired nucleotide changes.

All strains used were derivatives of W303. Epitope tags at the C terminus of the endogenous Scc1p were generated by a PCR one-step tagging method (W. Zacchariae and K.N., unpublished results). The Myc-epitope tag at the N terminus of endogenous Scc1 was obtained by integration of a N-terminally tagged portion of Scc1 at the *SCC1* locus. Strains expressing Scc1-Myc18, Esp1, and Cdc20 under control of the *GAL1-10* promoter have been described[7,12,21].

Cell-growth and cell-cycle experiments. Cells were grown in complete medium[34] at 25 °C unless otherwise stated. Strains expressing Cdc20, Esp1 or Scc1 from the *GAL1-10* promoter were grown in complete medium containing 2% raffinose as carbon source. The *GAL1-10* promoter was induced by adding 2% galactose. A G1-like arrest was achieved by adding 1 μg ml^{-1} of the pheromone α-factor to the medium. For metaphase arrest, 15 μg ml^{-1} nocodazole was added with 1% DMSO. Metaphase arrest due to Cdc20 depletion was obtained in cells with Cdc20 under control of the *GAL1-10* promoter by shifting to medium containing raffinose only. For release from the arrest, 2% galactose was added back to the culture.

***In vitro* assay for Esp1 activity.** A crude Triton X-100-insoluble chromatin preparation was obtained from yeast cells as described[19]. The pelleted chromatin was resuspended in yeast cell extracts that had been prepared similarly to the supernatant fraction of the chromatin preparation. Routinely, one tenth volume of an ATP regenerating system (50 mM HEPES/KOH, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 600 mM creatine phosphate, 1.5 mg ml^{-1} phophocreatine kinase, 1 mM DTT, 10% glycerol) was added. (This addition was later found not to be essential.) Reactions were incubated for 10 min at 25 °C with gentle shaking, and stopped on ice. The chromatin fraction was separated again from the supernatant by centrifugation, and resuspended in buffer EBX[19]. Equivalent aliquots of supernatant and chromatin pellet were analysed by SDS–PAGE and western blotting. HA-tagged Scc1 was detected using monoclonal antibody 16B12, Myc-tagged Scc1 with monoclonal antibody 9E10. As overexpression of Esp1 from the *GAL1-10* promoter is toxic to cells, extracts with overproduced Esp1 were prepared 2 h after induction with galactose of a culture pregrown in medium containing raffinose only.

Protein sequencing of the Scc1 cleavage site. The C-terminal Scc1 cleavage fragment was isolated from strain K7756 (*MATa cdc20Δ GAL-CDC20 SCC1-myc18*). Cells synchronized in anaphase were obtained as described for Fig. 3. Protein extract of 5×10^9 cells was prepared by breakage with glass beads in breakage buffer (50 mM HEPES/KOH, pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 0.25% Triton X-100, 0.1% SDS, plus protease inhibitors). Myc-epitope-tagged protein was immunoprecipitated with 20 μg anti-Myc 9E11 monoclonal antibody, resolved on SDS–PAGE and transferred to a PVDF membrane[35]. N-terminal sequencing of the band corresponding to the Scc1 cleavage fragment was performed using an Applied Biosystems 492A sequencer. The amino-acid sequence was RLGESIM, corresponding to the Scc1 amino-acid residues from position 269.

Chromosome spreading. Analysis of DNA content and chromosome spreading have been described[7], but spheroplastation to prepare cells for spreading was at 37 °C for only 5 min.

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1. Gimenez-Abian, J. F., Clarke, D. J., Mullinger, A. M., Downes, C. S. & Johnson, R. T. A postprophase topoisomerase II-dependent chromatid core separation step in the formation of metaphase chromosomes. *J. Cell. Biol.* 131, 7–17 (1995).
2. Losada, A., Hirano, M. & Hirano, T. Identification of *Xenopus* SMC protein complexes required for sister chromatid cohesion. *Genes Dev.* 12, 1003–1012 (1998).
3. Nicklas, R. B. The forces that move chromosomes in mitosis. *Annu. Rev. Biophys. Chem.* 17, 431–449 (1988).
4. Miyazaki, W. Y. & Orr-Weaver, T. L. Sister chromatid cohesion in mitosis and meiosis. *Annu. Rev. Genet.* 28, 167–187 (1994).
5. Rieder, C. L. & Salmon, E. D. The vertebrate cell kinetochore and its roles during mitosis. *Trends Cell Biol.* 8, 310–318 (1998).
6. Nasmyth, K. Separating sister chromatids. *Trends Biochem. Sci.* 24, 98–104 (1999).
7. Michaelis, C., Ciosk, R. & Nasmyth, K. Cohesins: Chromosomal proteins that prevent premature separation of sister chromatids. *Cell* 91, 35–45 (1997).
8. Guacci, V., Koshland, D. & Strunnikov, A. A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of *MCD1* in *S. cerevisiae*. *Cell* 91, 47–57 (1997).
9. Toth, A. *et al.* Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. *Genes Dev.* 13, 320–333 (1999).
10. Uhlmann, F. & Nasmyth, K. Cohesion between sister chromatids must be established during DNA replication. *Curr. Biol.* 8, 1095–1101 (1998).
11. Skibbens, R. V., Corson, L. B., Koshland, D. & Hieter, P. Ctf7p is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery. *Genes Dev.* 13, 307–319 (1999).
12. Ciosk, R. *et al.* An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* 93, 1067–1076 (1998).
13. May, G. S., McGoldrick, C. A., Holt, C. L. & Denison, S. H. The *bimB3* mutation of *Aspergillus* nidulans uncouples DNA replication from the completion of mitosis. *J. Biol. Chem.* 267, 15737–15743 (1992).
14. Funabiki, H., Kumada, K. & Yanagida, M. Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. *EMBO J.* 15, 6617–6628 (1996).
15. Nagase, T., Seki, N., Ishikawa, K., Tanaka, A. & Nomura, N. Prediction of the coding sequences of unidentified human genes. V. The coding sequences of 40 new genes (KIAA0161–KIAA0200) deduced by analysis of cDNA clones from human cell line KG-1. *DNA Res.* 3, 17–24 (1996).
16. Cohen-Fix, O., Peters, J.-M., Kirschner, M. W. & Koshland, D. Anaphase initiation in *Saccharomyces cervisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.* 10, 3081–3093 (1996).
17. Peters, J.-M. SCF and APC the Yin and Yan of cell cycle regulated proteolysis. *Curr. Opin. Cell Biol.* 10, 759–768 (1998).
18. Yamamoto, A., Guacci, V. & Koshland, D. Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). *J. Cell Biol.* 133, 99–110 (1996).
19. Liang, C. & Stillman, B. Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. *Genes Dev.* 11, 3375–3386 (1997).
20. Groigno, L. & Whitaker, M. An anaphase calcium signal controls chromosome disjunction in urchin embryos. *Cell* 92, 193–204 (1998).
21. Lim, H. H., Goh, P.-Y. & Surana, U. Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast. *Curr. Biol.* 8, 231–234 (1998).
22. McGrew, J. T., Goetsch, L., Byers, B. & Baum, P. Requirement for ESP1 in the nuclear division of *S. cerevisiae*. *Mol. Biol. Cell* 3, 1443–1454 (1992).
23. Rudner, A. D. & Murray, A. W. The spindle assembly checkpoint. *Curr. Opin. Cell Biol.* 8, 773–780 (1996).
24. Amon, A. The spindle checkpoint. *Curr. Opin. Gen. Dev.* 9, 69–75 (1999).
25. Funabiki, H. *et al.* Cut2 proteolysis required for sister-chromatid separation in fission yeast. *Nature* 381, 438–441 (1996).
26. Alexandru, G., Zachariae, W., Schleiffer, A. & Nasmyth, K. Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. *EMBO J.* 18, 2707–2721 (1999).
27. Melby, T. E., Ciampaglio, C. N., Briscoe, G. & Erickson, H. P. The symmetrical structure of structural maintenance of chromosomes (SMC) and MukB proteins: long, antiparallel coiled coils, folded at a flexible hinge. *J. Cell Biol.* 142, 1595–1604 (1998).
28. Akhmedov, A. T. *et al.* Structural maintenance of chromosomes protein C-terminal domains bind preferentially to DNA with secondary structure. *J. Biol. Chem.* 273, 24088–24093 (1998).
29. Lin, Y., Larson, K. L., Dorer, R. & Smith, G. R. Meiotically induced rec7 and rec8 genes of *Schizosaccharomyces pombe*. *Genetics* 132, 75–85 (1992).
30. Molnar, M., Bahler, J., Sipicki, M. & Kohli, J. The *rec8* gene of *Schizosaccharomyces pombe* is involved in linear element formation, chromosome pairing and sister-chromatid cohesion during meiosis. *Genetics* 141, 61–73 (1995).
31. Birkenbihl, R. P. & Subramani, S. The *rad21* gene product of *Schizosaccharomyces pombe* is a nuclear, cell cycle-regulated phosphoprotein. *J. Biol. Chem.* 270, 7703–7711 (1995).
32. Uzawa, S., Samejima, I., Hirano, T., Tanaka, K. & Yanagida, M. The fission yeast cut1+ gene regulates spindle pole duplication and has homology to the budding yeast ESP1 gene. *Cell* 62, 913–925 (1990).
33. Gietz, R. D. & Sugino, A. New yeast–*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six base pair restriction sites. *Gene* 74, 527–534 (1988).
34. Rose, M. D., Winston, F. & Hieter, P. *Laboratory Course Manual for Methods in Yeast Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1990).
35. Eckerskorn, C. & Lottspeich, F. Structural characterization of blotting membranes and the influence of membrane parameters for electroblotting and subsequent amino acid sequence analysis of proteins. *Electrophoresis* 14, 831–838 (1993).
36. Eddy, S. R. Hidden Markov models. *Curr. Opin. Struct. Biol.* 6, 361–365 (1996).
37. Kumada, K. *et al.* Cut1 is loaded onto the spindle by binding to Cut2 and promotes anaphase spindle movement upon Cut2 proteolysis. *Curr. Biol.* 8, 633–641 (1998).

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Disjunction of Homologous Chromosomes in Meiosis I Depends on Proteolytic Cleavage of the Meiotic Cohesin Rec8 by Separin

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Summary

It has been proposed but never proven that cohesion between sister chromatids distal to chiasmata is responsible for holding homologous chromosomes together while spindles attempt to pull them toward opposite poles during metaphase of meiosis I. Meanwhile, the mechanism by which disjunction of homologs is triggered at the onset of anaphase I has remained a complete mystery. In yeast, cohesion between sister chromatid arms during meiosis depends on a meiosis-specific cohesin subunit called Rec8, whose mitotic equivalent, Scc1, is cleaved at the metaphase to anaphase transition by an endopeptidase called separin. We show here that cleavage of Rec8 by separin at one of two different sites is necessary for the resolution of chiasmata and the disjunction of homologous chromosomes during meiosis.

Introduction

During mitosis, cohesion between sister chromatids generated during DNA replication (Uhlmann and Nasmyth, 1998; Skibbens et al., 1999; Toth et al., 1999) provides the means by which sister kinetochores attach to spindles that extend to opposite poles of the cell during prometaphase (Rieder and Salmon, 1998). When this occurs, sister chromatids come under tension as they are pulled in opposite directions (Nicklas, 1988). This tension lasts throughout metaphase, until sister chromatid cohesion is suddenly dissolved soon after congression of all chromosomes to the metaphase plate. Loss of cohesion triggers the segregation of sisters to opposite poles during anaphase (Nasmyth et al., 2000; Uhlmann et al., 2000 [this issue of *Cell*]).

During meiosis, two rounds of chromosome segregation following a single round of chromosome duplication give rise to haploid gametes from diploid germ cells. Remarkable changes in the behavior of chromosomes are required to produce this result. One is the pairing of homologous chromosomes and the subsequent recombination (or cross over) of the DNA strands and axes of homologous chromatids. Another is the monoorientation of sister kinetochores at the first meiotic division, which ensures their attachment to the same spindle pole and prevents the usual bipolar attachment. Due largely to these two properties, pairs of homologous chromosomes and not sister chromatids come under tension during metaphase of meiosis I, as kinetochores from homologs attach to spindles extending to opposite poles (Moore, 1998; Zickler and Kleckner, 1998; Orr-Weaver, 1999).

It has been proposed but never proven that sister chromatid cohesion within chromosome arms distal to chiasmata is responsible for holding homologs together until the onset of anaphase I (Maguire, 1974; Carpenter, 1994). If so, loss of this cohesion must be necessary for, and might even trigger, disjunction of homologs at the first meiotic division. Consistent with this hypothesis, sister chromatid arms remain closely connected throughout diakinesis and metaphase I, and invariably part from each other at the onset of anaphase I. Meanwhile, the segregation of sister chromatids at the second meiotic division depends on cohesion in the vicinity of their centromeres, which, unlike that along their arms, is maintained until metaphase of meiosis II and is dissolved only at the onset of anaphase II (Orr-Weaver, 1998).

Cohesion between sister chromatids during mitosis in yeast is mediated by a multisubunit complex called cohesin (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998; Nasmyth, 1999; Toth et al., 1999). The connections between sisters made by cohesin are dissolved at the onset of anaphase by proteolytic cleavage of one of its subunits, Scc1 (Uhlmann et al., 1999), by a specialized endopeptidase called separin, also known as Esp1 or separase (Uhlmann et al., 2000 [this issue of *Cell*]). Scc1 contains two sites recognized by separin and cleavage at either one of the two sites is necessary for sister separation. Remarkably, the best two matches to Scc1's separin cleavage sites within the entire yeast proteome are both found in the meiotic cohesin subunit Rec8 (Uhlmann et al., 1999), which is required for sister chromatid cohesion during meiosis but not mitosis (Molnar et al., 1995; Klein et al., 1999; Watanabe and Nurse, 1999). Rec8, along with other cohesin subunits, lines the entire longitudinal axes of pachytene chromosomes. It disappears from chromosome arms shortly before the first meiotic division, but persists in the vicinity of centromeres until the onset of anaphase II (Klein et al., 1999).

To address whether cleavage of Rec8 might be required for the resolution of chiasmata and hence, the disjunction of homologs, we generated noncleavable mutations in Rec8's potential separin cleavage sites. We show here that both sites are indeed substrates for separin in vitro and in vivo. We also show that their simultaneous mutation prevents Rec8's disappearance from chromosome arms during the first meiotic division without altering other meiosis I events, that the lack of

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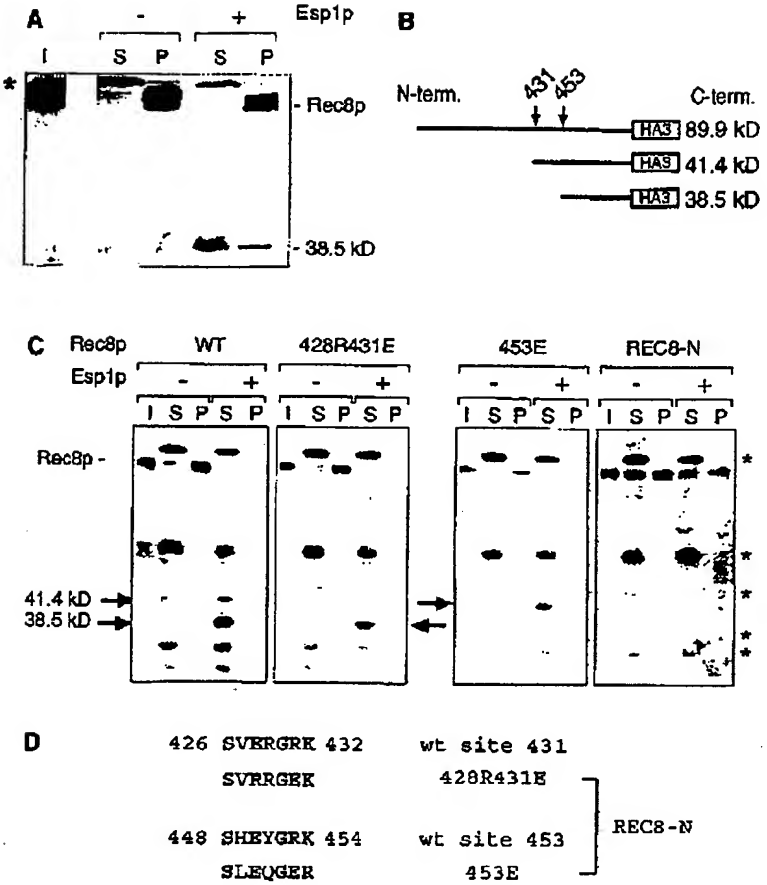

Figure 1. Rec8 Is Cleaved In Vitro by Separin at Residues 431 and 453

(A) Rec8 replacing Scc1 in mitotic cells is a substrate for separin cleavage in an in vitro reaction. A chromatin pellet was isolated from strain *K8811* (W303 prSCC1-REC8-HA3-LEU2::leu2 *scc1Δ*::URA3) and incubated in extracts of cells that had (lanes +) or had not (lanes) been induced to overexpress Esp1 (*K7287*: W303 *esp1-1*, trp1::TRP1 Gal-ESP1) (Uhlmann et al., 1999). S: supernatant (soluble fraction) isolated after the in vitro reaction by centrifugation. P: corresponding chromatin pellet. I: input, total amount of Rec8 binding the chromatin before the in vitro reaction. Asterisk: antibody cross-reacting band.

(B) Scheme of full-length Rec8 protein and putative cleavage site positions, with the expected cleavage product sizes.

(C) Six hours after the induction of meiosis, chromatin pellets were prepared from strains *K8806* (SK1 MATa/α REC8-HA3 wt -LEU2::*rec8Δ*::kanMX4), *K8968* (SK1 MATa/α REC8-HA3 428R431E-LEU2::*rec8Δ*::kanMX4), *K8805* (SK1 MATa/α REC8-HA3 453E-LEU2::*rec8Δ*::kanMX4), and *K8816* (SK1 MATa/α REC8-N-HA3-LEU2::*rec8Δ*::kanMX4) and incubated in extracts of cells that had (lanes +) or had not (lanes −) been induced to overexpress Esp1. S: supernatant. P: corresponding chromatin pellet. I: input. Asterisk: antibody cross-reacting bands.

(D) Amino acid changes in three Rec8 mutants. REC8-N is the mutant where both cleavage sites are modified simultaneously.

Rec8 cleavage blocks homolog disjunction, and that mutation of separin causes a similar phenotype.

Our observations imply that chiasmata are maintained until metaphase I by sister chromatid cohesion along chromosome arms, which is mediated by a version of cohesion complex containing Rec8, and is resolved at the onset of anaphase I by cleavage of Rec8 by separin. They also suggest that Rec8 in the vicinity of centromeres is protected from separin throughout the first meiotic division by an unknown mechanism. These results imply that proteolytic cleavage of Scc1-like cohesin subunits by separin may be a general mechanism for dissolving sister chromatid cohesion at metaphase to anaphase transitions.

Results

Mitotic Rec8 Is Cleaved by Separin In Vitro

To investigate whether Rec8 can serve as a substrate for separin, we took advantage of the discovery that Rec8 can substitute for Scc1 during vegetative growth. Rec8 is normally not produced during mitotic divisions and deletion of the *SCC1* gene is lethal at all temperatures (Uhlmann and Nasmyth, 1998). However, replacement of the *SCC1* gene by an HA-tagged version of *REC8* expressed from the *SCC1* promoter permits cells to proliferate at 25°C but not at 37°C (data not shown). Rec8 protein produced during mitotic divisions from this strain is cleaved by separin (Esp1) in vitro (Figure 1A). This suggests that Rec8 is a separin substrate, at least when presented on mitotic chromosomes.

Meiotic Rec8 Is Cleaved at Residues 431 and 453

To investigate whether meiotic Rec8 is also a separin substrate, we prepared chromatin from Rec8-HA3-expressing meiotic cells and treated it with extracts from mitotic cells. The chromatin was produced from cells that had completed premeiotic DNA replication but had not yet undergone the first meiotic division. Rec8-HA3 was both cleaved and removed from the pellet fraction by an extract from separin (Esp1) overproducing cells (Figure 1C, WT panel, lanes +) but not by one from *esp1-1* mutant cells (Figure 1C, WT panel, lanes −). Two different separin-dependent cleavage products (41.4 kDa and 38.5 kDa) were produced by this reaction (see Figures 1B and 1C, panel WT). Their sizes are consistent with cleavage at positions 431 and 453, the two sites within Rec8 closely related to Scc1 cleavage sites.

To confirm the identity of these cleavage sites, we mutated the proposed p1 arginine at site 453 to glutamic acid (Figure 1D, 453E). This mutation abolished production of the 38.5 kDa fragment (Figure 1C, panel 453E), suggesting that this product is generated by cleavage at position 453. An equivalent mutation of site 431 (431E) reduced but did not abolish production of the 41.4 kDa fragment (data not shown). We therefore produced a double mutant in which the p1 arginine at position 431 was replaced by glutamic acid and the conserved glu-

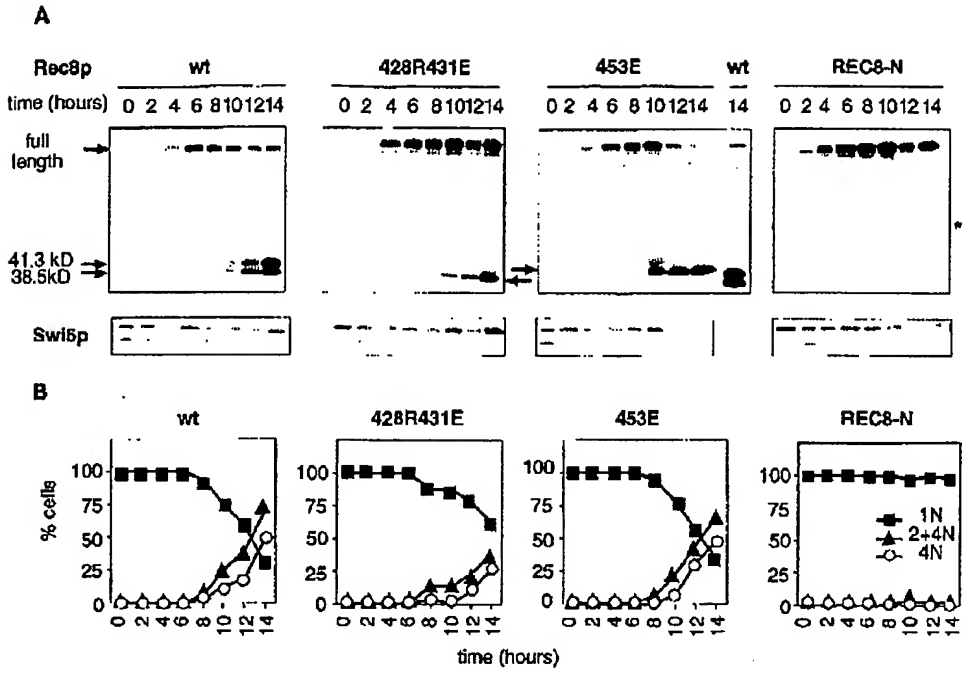

Figure 2. Meiotic Rec8 Is Cleaved In Vivo

(A) In vivo analysis of Rec8 cleavage during a meiotic time course for Rec8-HA3 wild type and the three mutants in *ubr1*Δ background. Both cleavage products (38.5 kDa and 41.4 kDa) are detectable around the first meiotic division in the wild type (strain *K9154:* SK1 MATa/α REC8-HA3 wt -LEU2::*rec8*Δ::kanMX4 *ubr1*Δ::TRP1). In the mutant 428R431E (strain *K9156*: SK1 MATa/α REC8-HA3 428R431E-LEU2::*rec8*Δ::kanMX4 *ubr1*Δ::TRP1) only the shorter product is generated (38.5 kDa), while the mutant 453E (strain *K9155*: SK1 MATa/α REC8-HA3 453E-LEU2::*rec8*Δ::kanMX4 *ubr1*Δ::TRP1) originates only the longer cleavage product (41.4 kDa). In this particular experiment, the kinetic of meiotic progression of strain *K9156* is slightly slower than the other strains. REC8-N (*K9157:* SK1 MATa/α REC8-N-HA3-LEU2::*rec8*Δ::kanMX4 *ubr1*Δ::TRP1) does not produce any of the expected cleavage products and full-length REC8-N protein levels do not decrease even at the latest stages. Swi6 was detected via specific antibody and used as loading control . Asterisk: antibody cross-reacting band.

(B) Analysis of nuclear division progression in DAPI-stained *ubr1*Δ cells allows detection of the effect of Rec8 mutant expression on meiotic progression. 1N: mononucleate cell percentage; 2N+4N: bi-tetranucleate cell percentage; 4N: tetranucleate percentage.

tamic acid at position 428 was replaced by arginine. This double mutation (Figure 1D, 428R431E) abolished production of the 41.4 kDa fragment but did not affect the 38.5 kDa fragment (Figure 1C, panel 428R431E). Mutation of both sites (Figure 1D, REC8-N) abolished production of both 38.5 kDa and 41.4 kDa fragments and prevented removal of Rec8 from the chromatin "pellet" fraction (Figure 1C, panel REC8-N). These data suggest that separin made by mitotic cells removes Rec8 from meiotic chromatin in vitro by cleavage at sites 431 and 453.

The 38.5 kDa and (to a lesser extent) 41.4 kDa cleavage products are also produced by meiotic cells expressing Rec8-HA3 (data not shown). However, neither product ever accumulates to high levels, which hampers their reliable detection by Western blotting. Both products have N-terminal lysines, which are destabilizing amino acids for N-end rule ubiquitination, and their abundance is greatly elevated (data not shown) by inactivating the "N-end rule" Ubr1 ubiquitin protein ligase (Varshavsky, 1997). Degradation of Scc1 cleavage products is also mediated by Ubr1 (H. Rao, F. U., K. N., and A. Varshavsky, unpublished data). Fortunately, cells lacking Ubr1 still undergo meiosis efficiently, albeit somewhat more slowly than wild type, and this permitted us to follow Rec8 cleavage as *ubr1*Δ mutant cells undergo meiosis. Both 38.5 kDa and 41.4 kDa products appeared at the onset of meiosis I, which coincides with the reduction of full-length Rec8 (compare Figures 2A and 2B, panel wt). A similar reduction in Rec8's abundance also takes place at the onset of meiosis I in *UBR1* cells (data not shown and Figure 4B, left panel). The 38.5 kDa fragment was abolished by the 453E mutation (Figure 2A, panel 453E) while the 41.4 kDa fragment was abolished by the 428R431E mutation (Figure 2A, panel 428R431E). Furthermore, mutation of both sites simultaneously (Figure 2A, panel REC8-N) abolished all cleavage and prevented the reduction of full-length Rec8, which normally occurs at the first meiotic division.

Separin Is Needed for Rec8 Cleavage, for Removal of Rec8 from Chromosomes, and for the First Meiotic Division

The disappearance of separin's inhibitor, securin (Pds1), at the onset of anaphase I, followed by its reappearance between divisions and disappearance once again at the onset of anaphase II (Salah and Nasmyth, 2000) suggests that separin might be transiently activated twice during meiosis: once shortly before the first meiotic division and a second time shortly before the second division. To investigate separin's role during meiosis, we isolated *esp1* DNAs from several *ts esp1* mutant strains and transferred them into the SK1 background (Experimental Procedures). All mutant strains sporulated effi-

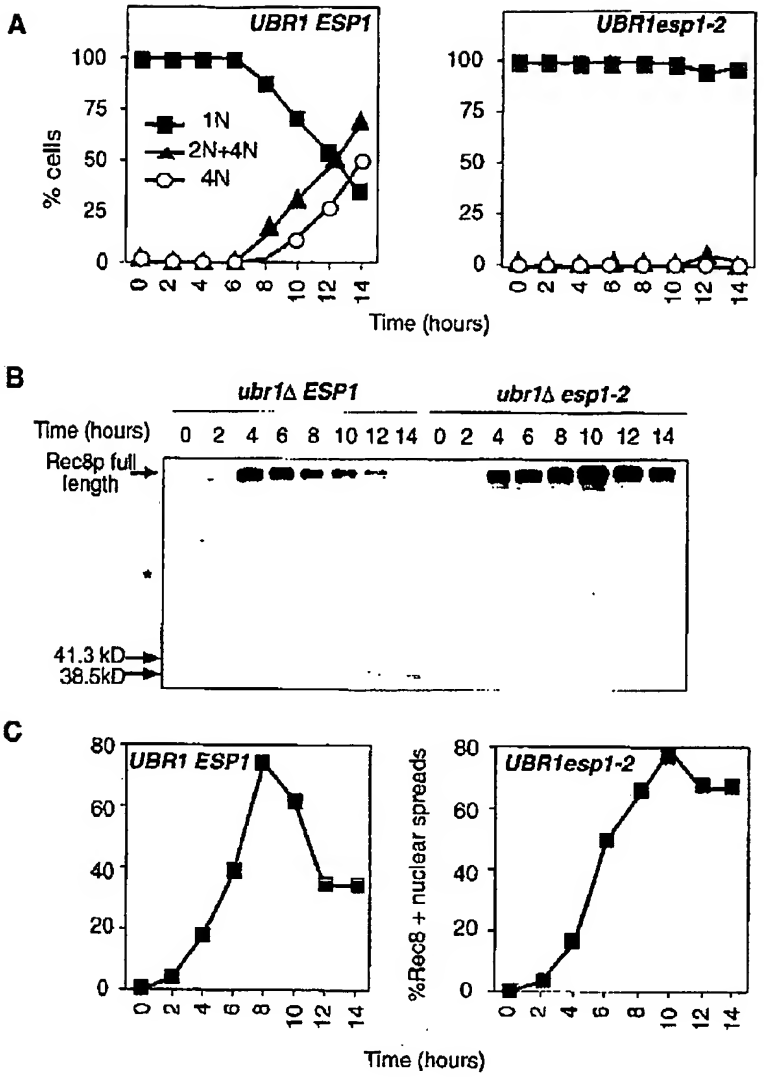

Figure 3. Rec8 Protein Level Decrease at Metaphase–Anaphase I Transition Requires In Vivo Separin Activity

(A) Nuclear division (scored by DAPI staining) is blocked at 34°C in strain *K8684* (SK1 MATα/a *UBR1 esp1-2* REC8-HA3 wt-LEU2::*rec8*Δ::kanMX4), carrying the thermosensitive *esp1* allele *esp1-2*. 1N: mononucleate cell percentage; 2N+4N: bi-tetranucleate cell percentage; 4N: tetranucleate percentage.

(B) Western blot analysis of Rec8-HA3 protein levels at 34°C in an *ESP1 ubr1*Δ (*K9154*) and in an *esp1-2 ubr1*Δ background (*K9158*: SK1 MATa/α *esp1-2* REC8-HA3 wt-LEU2-*rec8*Δ::kanMX4 *ubr1*Δ::TRP1). In *esp1-2*, full-length Rec8 accumulates even at the latest time points, while in the *ESP1* background, Rec8 protein levels start to decrease in correspondence of first meiotic division. Both cleavage products are generated in the wild type, while only a putative longer product is detectable at low levels after overexposure in *esp1-2*. Asterisk: antibody cross-reacting band.

(C) Chromosome spreads from strain *K8806* (*UBR1 ESP1*) and *K8684* (*UBR1 esp1-2*) sporulated at 34°C were immunostained for Rec8-HA3 and positive nuclei were counted for each time point.

ciently at 25°C but did so poorly, if at all, at 34°C (the highest temperature at which wild type sporulates efficiently). Some mutants, for example *esp1-2* and *esp1-4*, failed to sporulate at 34°C, whereas others, for example *esp1-1*, did so inefficiently and produced (at least on plates), a high proportion of two-spored asci (data not shown).

We analyzed in further detail the phenotype of an *esp1-2* diploid strain expressing an HA-tagged Rec8 protein. Premeiotic DNA replication, formation and dissolution of synaptonemal complex (monitored by Zip1 immunostaining of chromosome spreads [Sym et al., 1993; Dong and Roeder, 2000]), and assembly of meiosis I spindles (scored by in situ immunofluorescence using anti-tubulin antibody) took place with similar kinetics in wild-type and *esp1-2* cells at 34°C (data not shown). However, the mutant cells failed both to divide their nuclei (Figure 3A) and to elongate their spindles at the first division (data not shown). Western blotting showed that the disappearance of full-length Rec8 protein was greatly delayed and that the production of 38.5 kDa and 41.4 kDa cleavage fragments was greatly reduced in the mutant cells (Figure 3B). Furthermore, analysis of chromosome spreads immunostained for Rec8-HA3 showed that Rec8 failed to dissociate from chromosomes in *esp1-2* mutant cells (Figure 3C). Despite these defects, the lack of separin activity in *esp1-2* mutants does not seem to arrest the meiotic process, as the meiosis I spindles eventually break down and cells attempt to form two, albeit abnormal, meiosis II spindles and eventually form abnormal spores, which often lack DNA (data not shown). The mutant cells never form viable spores. Our data indicate that both Rec8's site-specific cleavage and its dissociation from chromosomes during meiosis I depend on separin activity.

Rec8 Cleavage Is Required for Homolog's Disjunction

To address whether the failure of separin mutants to undergo meiosis I might be due to their failure to cleave Rec8, we investigated the meiotic phenotypes of strains expressing cleavage site mutant proteins. Meiosis appeared unaffected by mutations that abolished cleavage solely at the 431 site (428R431E) or at the 453 site (453E) (Figure 2B, panels 428R431E and 453E), even when the mutations were homozygous; that is, when the mutant proteins were the only form of Rec8 made by the cell.

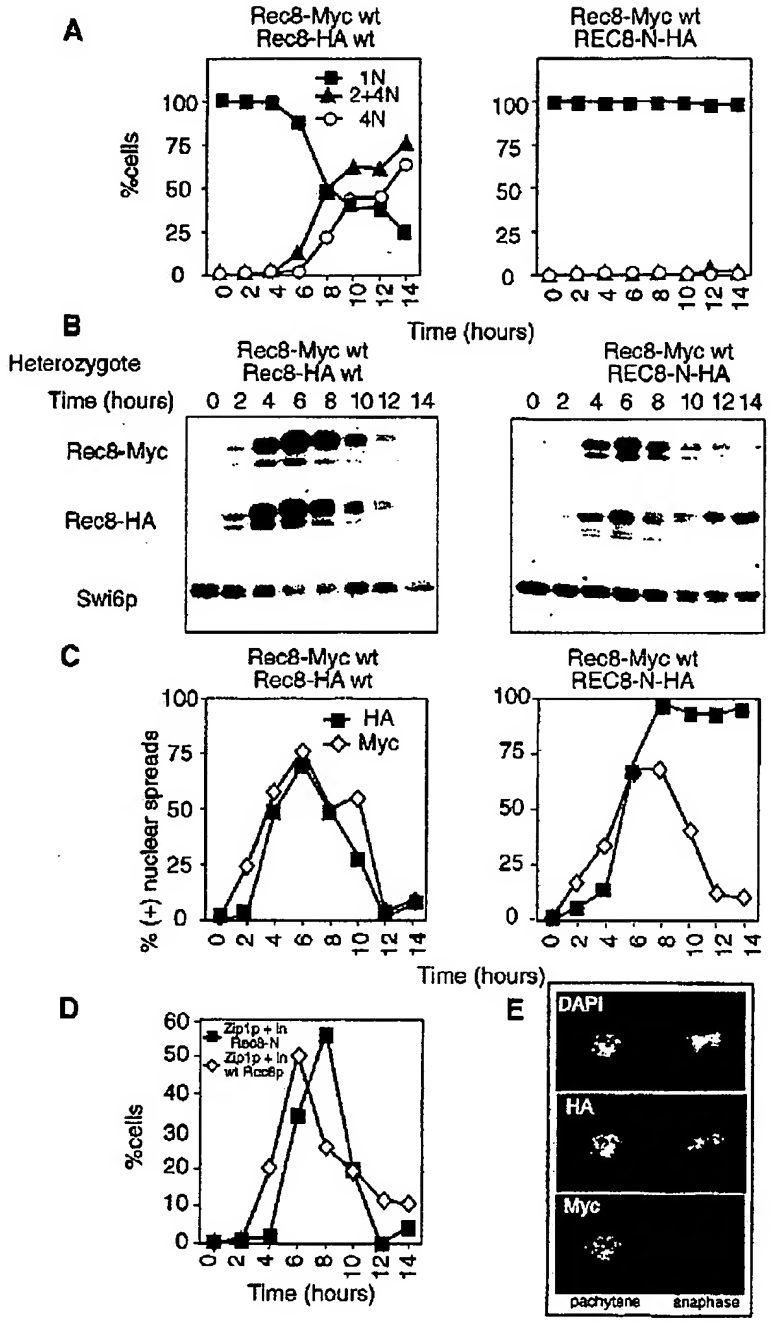

Figure 4. Uncleavable Rec8 (REC8-N) Causes a Dominant Segregation Block

(A) Nuclear division during a meiotic time course (scored by DAPI staining) in a strain heterozygous for wild-type Rec8 tagged with Myc and HA epitopes (*K8971*: SK1 MATa/α REC8 wt-Myc9::ura3 REC8-HA3 wt-LEU2::*rec8*Δ::kanMX4) and in a strain heterozygous for wild-type Rec8-Myc9 and REC8-N-HA3 (*K8970*: SK1 MATa/α, REC8wt-Myc9::ura3 REC8-N-H3-LEU2::*rec8*Δ::kanMX4). 1N: mononucleate cell percentage; 2N+4N: bi-tetranucleate cell percentage; 4N: tetranucleate percentage.

(B) Western blot analysis of Rec8 protein levels during a meiotic time course in strains *K8970* and *K8971*. Swi6 was used as loading control.

(C) Chromosome spreads were immunostained for Rec8-Myc9 wild type and for REC8-N HA3. Chromosomes positive for Myc and for HA were counted for each time point.

(D) Synaptonemal complex formation was assayed on chromosome spreads by immunostaining for Zip1 (see Experimental Procedures).

(E) Chromosome spreads on strain *K8970* were double-immunostained for Rec8-Myc9 wild type (FITC) and for REC8-N-HA3 (Cy3, see Experimental Procedures).

Neither "single" mutation had any significant effect on kinetics of meiotic divisions (Figure 2B), efficiency of sporulation, or viability of spores produced (data not shown). The somewhat sluggish division of the 453E mutant (Figure 2B, 453E) was not seen in cells with a wild-type *UBR1* gene and was probably due to slow entry into the meiotic program. In contrast, mutation of both sites (428R431E + 453E, called *REC8-N*) completely blocked both meiotic divisions (Figure 2B, panel REC8-N), even when heterozygous (i.e., when one *REC8* gene in diploids was mutant and the other was wild type; see Figure 4A). We conclude that proteolysis at either one of the two cleavage sites is both necessary and sufficient for meiotic chromosome segregation.

We next compared diploid cells in which a noncleavable Rec8 protein was tagged with HA epitopes and a wild-type Rec8 protein was tagged with Myc to diploid cells expressing HA- and Myc-tagged wild-type proteins. HA- and Myc-tagged wild-type proteins were degraded and disappeared from chromosomes with similar kinetics (Figures 4B and 4C, panels Rec8-Myc wt/Rec8-HA wt). In contrast, the noncleavable HA-tagged Rec8 failed both to be degraded and to dissociate from chromosomes, even when Myc-tagged wild-type protein expressed by the same cells had done so with normal kinetics (Figures 4B and 4C, panels Rec8-Myc wt/REC8-N-HA; Figure 4E). These data imply that cleavage of Rec8 is needed for its disappearance from chromo-

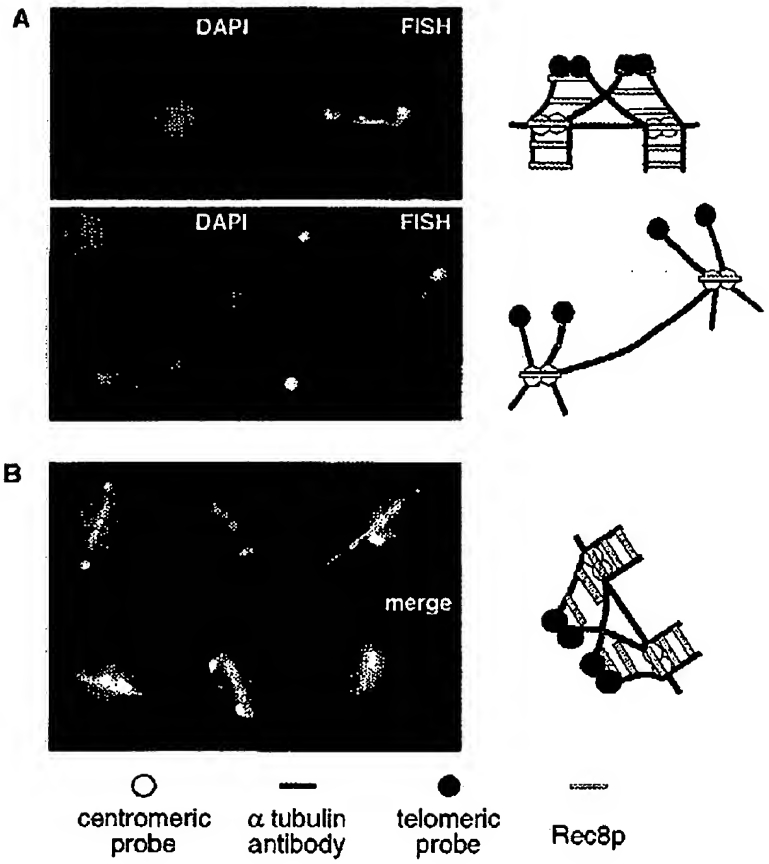

Figure 5. Uncleavable Rec8 Blocks Homologous Chromosomes' Disjunction

(A) FISH analysis of meiotic chromosome *XI* in a wild-type background (*K8806*) at 5 hr. The upper panel shows a metaphase I cell: the homologous centromeres (yellow signals) have already reached the spindle pole bodies, but the homologous telomeres still cluster in the medial region. The lower panel shows an anaphase I cell. Homologous telomeres are now separated. In addition, sister telomeres are apart too, confirming that homologs' separation is coincident with loss of cohesion along sister chromatid arms. A schematic model of this interpretation is drawn at the side of the pictures.

(B) FISH analysis of meiotic behavior of chromosome *XI* in a REC8-N background (*K8816*) at 12 hr. All cells show a single DNA mass (blue) or metaphase I or anaphase I A spindles. Homologous centromeres have separated (yellow signals), but telomeres (red signals) stay clustered.

some arms at the first meiotic division. However, the persistence of noncleavable Rec8 protein on chromosomes does not affect cleavage and dissociation of wild-type protein from the very same chromosomes. The presence of noncleavable protein also had little or no effect on the formation and dissolution of synaptonemal complexes, as monitored by Zip1 staining of chromosome spreads (Figure 4D), on formation of meiosis I spindles, or on the production of recombinant DNA molecules at the *LEU2* locus, which was measured using a diploid heterozygous at this locus for a restriction fragment polymorphism (data not shown) (Storlazzi et al., 1995; Xu and Kleckner, 1995).

Diploids expressing noncleavable Rec8-HA3 formed metaphase I spindles with similar kinetics to those expressing wild-type protein (data not shown). Fluorescence in situ hybridization (FISH) with probes specific for the centromere and telomere of the left arm of chromosome *XI* showed that centromeric regions of homologs had usually segregated to the poles in cells with metaphase spindles (Figure 5A, upper panel, yellow signals). In contrast, the distal portion of chromosome arms remained tightly associated, usually in the vicinity of the midline between the spindle poles (Figure 5A, upper panel, red signal). In wild-type cells, cohesion between sister chromatids along chromosome arms is lost soon after disjunction of homologs and extension of the meiosis I spindle (Figure 5A, lower panel, red signals). However, this process did not take place in cells expressing noncleavable Rec8. While centromeric regions (yellow) usually segregated to the spindle poles, sister chromatid arms not only remained paired but also failed to disjoin from their homologous partner (Figure 5B, single red signal). In summary, the phenotype of cells expressing noncleavable Rec8 (even in the presence of wild-type protein) resembles that of cells lacking separin activity. This raises the possibility that one, if not the only, crucial function of separin during meiosis I is to cleave Rec8, either at site 431 or at site 453.

Mutation of *SPO11* Restores Meiosis I in *esp1-2* Mutants and in Mutants Expressing Noncleavable Rec8

If cleavage of Rec8 by separin were necessary for separating sister chromatid arms and thereby for resolving chiasmata, then the lack of chromosome segregation at meiosis I in mutants either lacking separin activity or expressing noncleavable Rec8 should be suppressed by eliminating recombination. To test this, we analyzed the consequences of deleting *SPO11*. The endonuclease encoded by this gene generates the double strand breaks that initiate recombination during prophase (Bergerat et al., 1997; Keeney et al., 1997). *spo11Δ* mutants neither pair nor synapse homologous chromosomes during pachytene (Giroux et al., 1989; Loidl et al., 1994; Weiner and Kleckner, 1994; Rockmill et al., 1995; Cha et al., 2000) nor produce the chiasmata that hold homologs together during their alignment on the metaphase I spindle. Despite this crucial deficiency, *spo11Δ* mutants nevertheless form an apparently normal meiosis I spindle

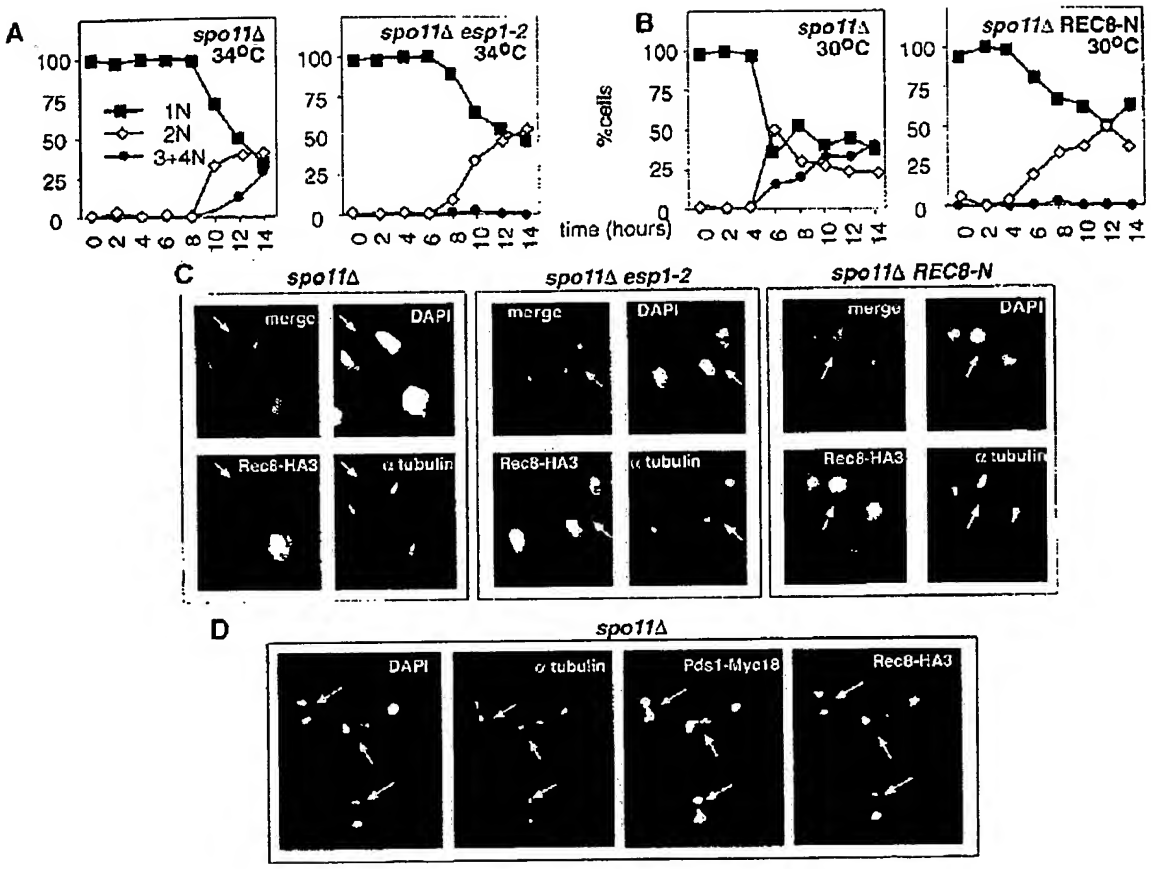

Figure 6. *spo11Δ* Rescues First Nuclear Division Block in REC8-N and in *esp1-2* Background

(A) The left panel shows progression of nuclear division (scored by DAPI staining of cells) in a *spo11Δ ESP1* background (*K8975*: SK1 MATa/α *spo11Δ*:: ura3 REC8-HA3 wt-LEU2::*rec8Δ*::kanMX4) at 34°C. The right panel shows the same in *spo11Δ esp1-2* background (*K8976*: SK1 MATa/α *esp1-2 spo11Δ*::URA3 REC8-HA3wt-LEU2::*rec8Δ*::kanMX4). 1N: mononucleate cell percentage; 2N+4N: bi-tetranucleate cell percentage; 4N: tetranucleate percentage.

(B) The left panel shows nuclear division progression (scored by DAPI staining) in a *spo11Δ REC8* background (*K8980*: SK1 MATa/α *spo11Δ*::URA3 heterozygous REC8-HA3 wt-LEU2::*rec8Δ*::kanMX4, *rec8Δ*::kanMX4) at 30°C. The right panel shows the same in *spo11Δ REC8-N* (*K8979*: SK1 MATa/α *spo11Δ*::URA3 heterozygous REC8-N-HA3-LEU2::*rec8Δ*::kanMX4, *rec8Δ*::kanMX4). 1N: mononucleate cell percentage; 2N+4N: bi-tetranucleate cell percentage; 4N: tetranucleate percentage.

(C) In situ immunofluorescence to visualize Rec8-HA3 (mouse anti-HA 16B12, see Experimental Procedures) and spindles (rat α tubulin, see Experimental Procedures). The left panel shows a metaphase II cell (indicated by the arrow) in *spo11Δ* (*K8975*); at this stage, the majority of the cells (71%) retain only centromeric Rec8 (see Rec8-HA3 panel). In *spo11Δ esp1-2* (*K8976*, central panel) the arrow indicates metaphase II cells still positive for Rec8 staining (89.7% of the cells, see Rec8-HA3 panel). The right panel shows a metaphase II cell (indicated by the arrow) in *spo11Δ REC8-N* (*K8979*, right panel); 94% of metaphase II cells (one example is indicated by the arrow) are still positive for Rec8 staining (see Rec8-HA3 panel)

(D) Pds1-Myc18 levels were scored by in situ immunofluorescence (FITC, see Experimental Procedures) in a *spo11Δ* background (right panel, *K9096*: SK1 MATa/α *spo11Δ*:: ura3 REC8-HA3 wt-LEU2::*rec8Δ*::kanMX4, heterozygous for PDS1-Myc18::TRP1). The arrows indicate anaphase I cells. Rec8-HA3 (Cy3, see Experimental Procedures) and α tubulin staining (Cy5, see Experimental Procedures) are shown too.

and segregate homologous chromosomes at random to the two spindle poles (Klapholz et al., 1985). Having segregated homologs at random at the first meiotic division, *spo11Δ* mutants proceed with an apparently normal second meiotic division, during which they segregate sister chromatids to opposite poles.

We found that deletion of *SPO11* fully relieved the lack of chromosome segregation during meiosis I of mutants either lacking separin activity (*esp1-2*) (compare Figures 3A and 6A, right panels) or expressing noncleavable Rec8 protein (REC8-N) (compare Figure 2B, REC8-N panel and Figure 6B, right panel). FISH with probes for the centromere and left telomere of chromosome *XI* confirmed that *spo11Δ esp1-2* and *spo11Δ REC8-N* double mutants segregated homologs randomly during meiosis I with efficiencies and kinetics that resembled that of *spo11Δ* single mutants (data not shown). However, despite forming meiosis II spindles, the double mutants neither separated sister chromatids nor segregated chromosomes during what should have been meiosis II.

Deletion of *SPO11* did not, however, relieve the failure of *esp1-2* or *REC8-N* mutants to remove Rec8 from chromosomes at the first meiotic division. In situ immunofluorescence of fixed cells shows that Rec8-HA3 is reduced in abundance in most (i.e., 71%) *spo11Δ* metaphase II cells and is predominantly associated with centromeric regions (Figure 6C, left panel). In contrast, high

levels of Rec8 persisted throughout the nuclei of most metaphase II *spo11Δ REC8-N* (94%) (Figure 6C, right panel) and *spo11Δ esp1-2* (89.4%) (Figure 6C, central panel) double mutant cells.

During these studies, we discovered that most (75%) *spo11Δ* cells undergo the first meiotic division before destruction of Pds1 securin (Figure 6D). Furthermore, FISH analysis showed that sister telomeres separated during anaphase I in no more than 23% of *spo11Δ* mutant cells (data not shown). This contrasts with wild-type cells in which meiosis I is invariably associated with loss of arm cohesion (see Figure 5A) and where securin destruction always precedes the onset of anaphase I (Salah and Nasmyth, 2000). Because securin is a potent inhibitor of separin, this finding confirms that cleavage by separin is only needed for disjoining homologs if chiasmata have previously been produced by Spo11. Finally, our experiments on *spo11Δ* mutants imply that the failure of *esp1-2* and *REC8-N* mutants to segregate homologs at meiosis I cannot be due to a defective meiosis I spindle.

Discussion

It has long been suspected that sister chromatid cohesion along chromosome arms might have a crucial role in holding homologous chromosomes together following reciprocal exchange between maternal and paternal chromatids (Moore and Orr-Weaver, 1998). Cytological studies in a wide variety of organisms have shown that sister chromatids remain tightly paired throughout diakinesis and metaphase I but suddenly separate at the onset of anaphase I. Loss of cohesion along chromosome arms might even be the trigger that resolves chiasmata and thereby promotes segregation of homologous chromosomes to opposite poles at the first meiotic division. This hypothesis has, however, been difficult if not impossible to test in a rigorous manner without knowing about the molecules that mediate sister chromatid cohesion during meiosis I.

The starting point of the work described in this paper was the recent recognition that meiotic sister chromatid cohesion depends on a meiotic variant of the cohesin complex required for mitotic sister chromatid cohesion. Sister separation during mitosis depends on cleavage of cohesin's Scc1 subunit (Uhlmann et al., 1999) by an endopeptidase called separin or separase. During meiosis, Scc1 is replaced by a variant called Rec8 (Klein et al., 1999), which contains two potential separin cleavage sites. Our current work shows that both of these sites are indeed recognized by separin in vitro and are cleaved around the time of the first meiotic division in vivo. Mutant diploids expressing *REC8* genes lacking either one or the other cleavage site undergo meiosis normally but diploids expressing even only a single copy of a *REC8-N* gene (coding for uncleavable Rec8) fail to segregate chromosomes at either division, despite forming normal-looking meiotic spindles. The phenotype of these mutants resembles that of separin (*esp1-2*) mutants, which also fail to resolve chiasmata at meiosis I. The lack of chromosome segregation during meiosis I due to the expression of noncleavable Rec8 or due to separin inactivation is largely if not completely bypassed by eliminating recombination through deletion of the *SPO11* gene. Indeed, anaphase I takes place precociously in *spo11Δ* mutants and does so in the presence of high levels of separin's inhibitor, securin (Pds1). Thus, Rec8 cleavage and separin activity are only required for chromosome segregation during meiosis I if maternal and paternal chromatids have recombined and formed chiasmata. The simplest interpretation of these results is that sister chromatid cohesion (mediated by a Rec8-containing cohesin complex) distal to crossovers does indeed hold homologs together during metaphase of meiosis I and that this chiasmata linkage is resolved by cleavage of Rec8 by separin (Figure 7).

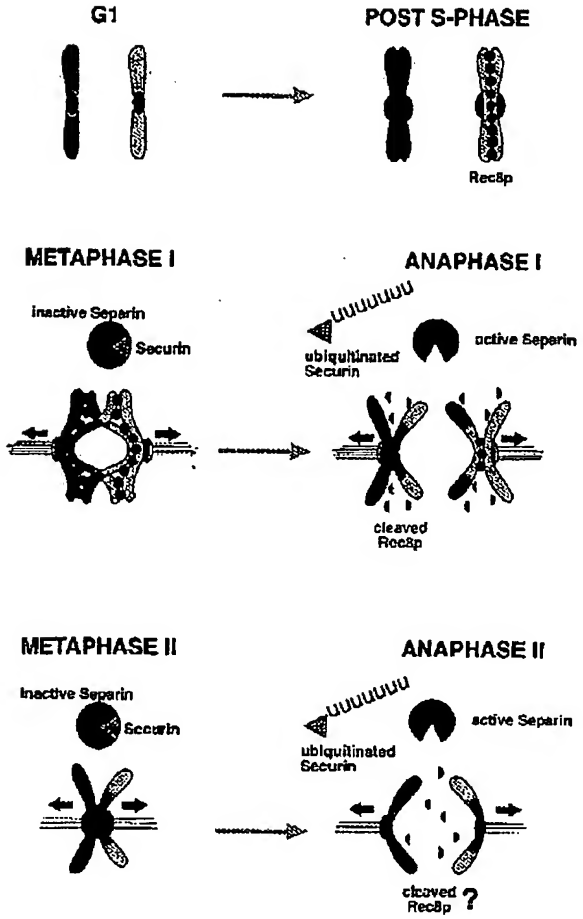

Figure 7. Separin-Dependent Rec8 Cleavage Is Required for First Meiotic Division

Sister chromatid cohesion is established during S-phase. During prophase, recombination takes place. As a consequence of this process, homologous chromosomes are held together from the sister chromatid cohesion complex (red spheres). At metaphase I, the securin Pds1 (green triangle) is ubiquitinated in a Cdc20-dependent manner. Its degradation activates the separin Esp1 (blue sphere). Esp1 cuts Rec8 in a site-specific manner along the arms, leaving homologous chromosomes free to move towards the opposite pole of the spindle. Centromeric Rec8 is protected from the cleavage by an unknown mechanism and persists at the centromere until metaphase–anaphase II transition. The mechanism of Rec8 removal from the centromere is still unknown; it is reasonable to hypothesize the involvement of Esp1 in the metaphase–anaphase II transition, too.

Rec8 Cleavage by Separin?

The crux of this paper is the claim that Rec8 is cleaved by separin during meiosis I. The evidence can be sum-

marized as follows: (1) Rec8 protein is degraded around the time of meiosis I. (2) Rec8 contains two sites with strong resemblance to Scc1's separin cleavage sites (indeed, these are the best two matches in the entire yeast proteome). (3) Both sites are indeed cleaved by separin in vitro, when presented either on mitotic or meiotic chromosomes. (4) Rec8 cleavage products are produced in large amounts around the time of Rec8 degradation during meiosis I. (5) Mutation of each site individually blocks cleavage at that site but not at the other, whereas mutation of both sites blocks degradation of the mutated Rec8 but not wild-type protein expressed in the same cell. (6) Separin mutants also fail to cleave and degrade Rec8 at meiosis I. (7) Securin (Pds1), which is known to inhibit separin activity, is degraded at around the same time that Rec8 degradation commences, shortly before the onset of anaphase I. (8) In the absence of recombination, cells undergo the first meiotic division prematurely and do so in the presence of high levels of securin. These data are all consistent with the notion that Rec8 degradation during meiosis I is due to cleavage by separin, which is activated by the destruction of securin. The products of Rec8 cleavage never accumulate to high levels in a *UBR1* background but are stabilized in *ubr1*Δ mutant cells, indicating that Rec8 cleavage products are rapidly targeted to the 26S proteosome destruction via the N-end rule ubiquitination pathway.

Rec8 Cleavage by Separin Is Needed for Chromosome Segregation

Expression of a form of Rec8 mutated at both cleavage sites (428R431E + 453E, called REC8-N) completely blocks meiotic chromosome segregation even when cells express equal amounts of wild-type Rec8 protein. A crucial question is whether this dominant phenotype is due to a lack of proteolytic cleavage or to an unforeseen side effect on an as yet unknown function of Rec8. Rec8 does indeed have multiple functions. It is required for sister chromatid cohesion, for the formation of synaptonemal complex (Klein et al., 1999; Parisi et al., 1999), for efficient recombination between homologs (Ponticelli and Smith, 1989; Krawchuk et al., 1999), and possibly even for rapid premeiotic DNA replication (Cha et al., 2000). *REC8-N* 's complete dominance contrasts with the lack of any phenotype caused by mutation of each "cleavage" site individually even when these single mutations are homozygous. Both the dominance of *REC8-N* and the silence of single site mutations are readily explained if the function of the mutated sequences is merely to serve as cleavage sites and if cleavage at either one of the two sites is necessary to destroy the cohesin connections that link sister chromatids. In contrast, neither phenomenon can be readily explained by the notion that the two sequences are in fact required for some other Rec8 activity needed for chromosome segregation. As far as we can tell, no function of Rec8 other than its susceptibility to separin cleavage is detectably altered by the *REC8-N* mutation. We therefore suggest that the main if not sole effect of the *REC8-N* mutation is to prevent proteolytic cleavage of those molecules expressed from the mutant locus and that these molecules persist in holding sister chromatids together even when an equivalent number of wild-type Rec8 molecules are degraded on schedule.

Mutation of both Rec8 cleavage sites has at least three effects on chromosome behavior. In wild-type cells, dissociation of Rec8 from chromosome arms, chiasmata resolution, and loss of arm sister chromatid cohesion all occur around the time that cells extend meiosis I spindles. However, none of these three events take place in cells that express noncleavable Rec8 nor, indeed, in *esp1-2* mutant cells.

Neither *REC8-N* nor the *esp1-2* mutations affect the attachment of sister centromeres to meiotic spindles and the segregation of maternal and paternal centromere pairs to opposite poles. In contrast, both mutations prevent the disjunction of homologous arm sequences. Disjunction of centromeres, but not of chromosome arms, suggests that mutants expressing REC8-N cannot resolve chiasmata. This suggests that the lack of chromosome segregation cannot be attributed to a defect in spindle function. Indeed, when recombination is eliminated by deleting *SPO11*, the spindles of *REC8-N* and *esp1-2* mutant cells are capable of segregating homologous chromosomes to each pole, albeit in a random manner. We therefore propose that the resolution of chiasmata (at least in yeast) is mediated by cleavage of Rec8 by separin. Rec8 is necessary for sister chromatid cohesion during meiosis and can even substitute Scc1 in this function during mitosis. It is therefore reasonable to suppose that homologs are held together from prophase until metaphase I primarily, if not exclusively, by cohesion between sisters distal to crossovers, which is mediated by a Rec8-containing cohesin complex. If so, cleavage of Rec8 by separin would trigger homolog disjunction by destroying sister chromatid cohesion. We cannot, however, exclude at this juncture the alternative possibility that the Rec8 cohesin complex mediates the linkage between homologs by participating in a special structure (Maguire's chiasma binder [Maguire, 1974]), which is situated not on chromosome arms but at crossover sites themselves.

Is Cleavage of Rec8 along Chromosome Arms Needed for Meiosis I in Animal Cells?

There is currently some uncertainty whether cleavage of Scc1 during mitosis triggers the separation of sister chromatids at the metaphase to anaphase transition in animal cells. Cleavage of something (possibly of residual cohesins) is presumably required because separin is conserved in all eukaryotes, but it is still unclear whether Scc1 is its target. The reason for this uncertainty is that the bulk of cohesin dissociates from animal cell chromosomes during prophase and prometaphase (Losada et al., 1998) and only small amounts remain associated with metaphase chromosomes (Waizenegger et al., 2000 [this issue of *Cell*]). Furthermore, when securin destruction is inhibited either by triggering the Mad2 chromosome alignment surveillance mechanism (Rieder and Palazzo, 1992) or by inactivating APC/cdc20 (Rieder and Cole, 1999), chromosome arms, though not centromeres, fully separate, presumably in the absence of any separin activity (Nasmyth et al., 2000). The implication is that sister chromatid cohesion along chromosome arms, though possibly not that at centromeres, can be dissolved by dissociation of cohesin from chromosomes by a process that does not involve proteolysis of Scc1. This raises the question whether a similar separin-inde-

pendent pathway exists in meiotic cells and, if so, whether it and not separin mediates homolog disjunction during meiosis I. It will therefore be of some interest to establish whether cleavage of Rec8 by separin is also needed for meiosis I in animal cells.

What Is the Fate of Rec8 at Centromeres?

Our data suggest that the bulk of Rec8 is cleaved at the first meiotic division and that this event triggers Rec8's dissociation from chromosome arms. Meanwhile, a small pool of Rec8 protein persists in the vicinity of centromeres until the second meiotic division and disappears at the onset of anaphase II (Molnar et al., 1995; Klein et al., 1999; Watanabe and Nurse, 1999). The fraction of Rec8 persisting at centromeres is too low and the synchrony of meiosis too poor to address directly whether cleavage of centromeric Rec8 is delayed until anaphase II. However, persistence on chromosomes very possibly reflects lack of cleavage. In which case, we propose that Rec8 protein in the vicinity of centromeres is specifically protected from separin during the first meiotic division, loses this protection soon after the reaccumulation of securin following meiosis I, and is cleaved by separin upon securin's destruction at the onset of anaphase II (Figure 7). Because cleavage of Scc1 is sufficient to trigger anaphase during mitosis (Uhlmann et al., 2000 [this issue of *Cell*]), we suggest that cleavage of Rec8 on chromosome arms triggers anaphase I, whereas cleavage of Rec8 at centromeres triggers anaphase II. Indeed, the notion that both meiotic divisions might be triggered by the same enzyme, namely separin, is consistent with observations in grasshoppers, showing that meiosis I bivalents transferred to the spindles of meiosis II cells disjoin at the same time as endogenous sister chromatids and that sister chromatids from meiosis II cells separate at the same time as meiosis I bivalents when transferred to the spindles of meiosis I cells (Nicklas, 1977).

Cleavage of Cohesins by Separins: A General Mechanism for Triggering Sister Separation

Despite the conservation of separins and Scc1-like cohesin subunits, there has thus far been no direct evidence that proteolytic cleavage of cohesin subunits might be a universal mechanism for separating sister chromatids. It has been hard to spot separin cleavage sites in animal Scc1 proteins. Furthermore, cohesin's dissociation from chromosomes during prophase in animal cells suggests that only small amounts remain on metaphase chromosomes where they might be subject to cleavage by separin. The discovery that cleavage of Rec8 is crucial for the resolution of chiasmata therefore represents concrete evidence that cleavage of cohesins by separin might be a universal mechanism for separating sister chromatids at the metaphase to anaphase transition. Most, if not all, the major hallmarks of meiotic cell divisions are conserved among eukaryotic organisms. In which case, the use of Scc1 and Rec8 cleavage to separate sister chromatids during mitosis and meiosis, respectively, may have existed in the common ancestor of all meiotic organisms.

Experimental Procedures

Plasmids and Yeast Strains

REC8-HA3 gene and its promoter (333 bp upstream of start codon) were amplified from genomic DNA of strain K8033 (SK1 MATa/α REC8-HA3::URA3) (Klein et al., 1999) and were cloned into YIplac128 (Gietz and Sugino, 1988). All *REC8* mutants were obtained by exchanging restriction fragments from *REC8* (NcoI-BglII sites) with PCR fragments obtained using primers containing the desired nucleotide changes.

All strains are derivatives of SK1, *rec8Δ* (K8079 SK1 MATa and K8081 MATα, *rec8Δ::kanMX4*) (Klein et al., 1999). After linearization, using the MluI site within the promoter, the constructs were integrated into the *REC8* locus, upstream of the deletion. Diploids were obtained by performing independently the integration into MATα and MATa strains and subsequently crossing them, or by diploidizing by transforming the haploid with an *HO*-expressing plasmid.

The *ubr1Δ* was obtained by PCR-mediated gene replacement (Wach et al., 1994), replacing the complete sequence of the ORF (positions +20 to 23 bp upstream the stop codon) with the *TRP1* marker.

The *esp1-2* allele was recovered from strain K8493 (*esp1-2*) using a gap repair strategy (Guthrie and Fink, 1991). The recovered allele was subcloned into the URA3-integrating vector pRS306 (Sikorski and Hieter, 1989). Following plasmid linearization the allele was transferred into the SK1 strain K8812 (SK1 MATα REC8-HA3 wt-LEU2::*rec8Δ*::kanMX4) by transformation and 5-FOA counter-selection (Guthrie and Fink, 1991). The resultant temperature-sensitive strain K8874 (SK1 MATα *esp1-2* REC8-HA3 wt-LEU2::*rec8Δ*::kanMX4) was diploidized by transformation with a plasmid containing the *HO* gene, generating the *esp1-2* homozygous diploid K8684. Transformation with a plasmid containing the wild type *ESP1* gene (c3944) rescued both the mitotic and meiotic temperature-sensitivity of this strain.

Sporulation Procedures

Strains were streaked onto YPG (glycerol) plates from stocks in 15% glycerol stored at −80°C and grown for 60 hr at 25°C. A single colony was patched on YPD and grown for 48 hr. Cells were inoculated in liquid YEPA 2% (2% bactopeptone, 1% yeast extract, and 2% potassium acetate) and grown for 10 hr to stationary phase, then inoculated into YEPA 1% (2% bactopeptone, 1% yeast extract, and 1% potassium acetate) and grown overnight, to ~3 OD/ml. They were subsequently washed with potassium acetate 2% (SPO medium) and incubated for 14–24 hr in SPO medium to a density of ~3–5 OD/ml. All the meiotic experiments were conducted at 30°C, with the exception of *esp1-2* experiments (shifted at 34°C after 2 hr in SPO medium at 25°C).

Chromosome Spreading

Chromosome spreading was performed according to procedures described previously (Nairz and Klein, 1997; Loidl et al., 1998). To detect Rec8-HA3, mouse 12CA5 or 16B12 (Babco) antibodies were used at 1:1200 and 1:600. The secondary antibody was anti-mouse Cy3 at 1:1000 (CHEMICON). Zip1 has been detected by rabbit antibody kindly provided by Shirleen Roeder. Goat anti-rabbit FITC 1:100 (CHEMICON) was used as secondary antibody. Rec8-Myc9 was detected by rabbit anti-Myc 1:200 (Gramsch) and goat anti-rabbit-FITC 1:50 (CHEMICON).

In Vitro Assay for Rec8 Cleavage by ESP1

In vitro cleavage of Rec8 was performed as described for Scc1 (Uhlmann et al., 1999). The chromatin substrate was prepared from 40–60 OD of meiotic cells after 6 hr in SPO medium.

Western Blotting

Cell extracts were prepared by cell breakage with glass beads into 2× protein loading buffer, preceded and followed by 5 min boiling. Equal amounts of protein were analyzed by SDS-PAGE and blotting according to standard procedures (Sambrook et al., 1989). The HA-epitope tag was detected by 16B12 mouse antibody 1:10000 and the Myc-tag was detected by mouse 9E10 antibody 1:200. Rabbit anti-Swi6 antibody has been diluted 1:100000 (Klein et al., 1999).

In Situ Immunofluorescence

The in situ immunostaining was performed according to Piatti et al. (1996). α-tubulin staining was obtained using a rat antibody (Serotec) 1:100. The secondary antibody was either goat anti-rat FITC 1:100 or donkey anti-rat Cy5 (CHEMICON) 1:50. Pds1-Myc18 was detected by rabbit anti-Myc 1:200 (Gramsch) and goat anti-rabbit-FITC 1:50 (CHEMICON).

Immunostaining and FISH

Cells were prepared according to the semispreading procedure described previously in Jin (2000). Spindles were immunolabeled with YOL1/34 monoclonal rat anti-yeast tubulin antibody (Kilmartin et al., 1982) (Serotec) and FITC-conjugated secondary antibody (SIGMA) according to a standard protocol (Pringle et al., 1991). For subsequent fluorescent in situ hybridization (FISH), the cells were postfixed for 10 min in 4% paraformaldehyde at room temperature. For labeling the centromeric region of chromosome XI, two overlapping cosmid clones (pUKG041, pEKG021) covering a 47 kb tract (including the centromere) were chosen. The telomere of the left arm of the same chromosome was marked by two overlapping cosmid clones (pUKG040, pEKG086) covering a region of 48 kb three kilobase pairs away from the physical end. All cosmid clones were kindly provided by Bernard Dujon (Thierry et al., 1995). The centromeric probe was labeled with Cy5-dUTP (Amersham) and the telomere-proximal probe with Cy3-dUTP (Amersham) using a standard nick translation protocol (see Loidl et al., 1998). FISH was performed according to Jin (2000).

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References

Bergerat, A., de Massy, B., Gadelle, D., Varoutas, P.C., Nicolas, A., and Forterre, P. (1997). An atypical topoisomerase II from Archaea with implications for meiotic recombination. Nature *386*, 414–417.

Carpenter, A.T. (1994). Chiasma function. Cell *77*, 957–962.

Cha, R.S., Weiner, B.M., Keeney, S., Dekker, J., and Kleckner, N. (2000). Progression of meiotic DNA replication is modulated by interchromosomal interaction proteins, negatively by Spo11 and positively by Rec8. Genes Dev. *14*, 493–503.

Dong, H., and Roeder, G.S. (2000). Organization of the yeast Zip1 protein within the central region of the synaptonemal complex. J. Cell Biol. *148*, 417–426.

Gietz, R.D., and Sugino, A. (1988). New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene *74*, 527–534.

Giroux, C.N., Dresser, M.E., and Tiano, H.F. (1989). Genetic control of chromosome synapsis in yeast meiosis. Genome *31*, 88–94.

Guacci, V., Koshland, D., and Strunnikov, A. (1997). A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in *S. cerevisiae*. Cell *91*, 47–57.

Guthrie, C., and Fink, G.R. (1991). Guide to Yeast Genetics and Molecular Biology. Vol. *194*, 281–302.

Jin, Q.-W. Fuchs., J., and Loidl, J. (2000). Centromere clustering is a major determinant of yeast interphase nuclear organization. J. Cell Sci., in press.

Keeney, S., Giroux, C.N., and Kleckner, N. (1997). Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. Cell *88*, 375–384.

Kilmartin, J.V., Wright, B., and Milstein, C. (1982). Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. J. Cell Biol. *93*, 576–582.

Klapholz, S., Waddell, C.S., and Esposito, R.E. (1985). The role of the SPO11 gene in meiotic recombination in yeast. Genetics *110*, 187–216.

Klein, F., Mahr, P., Galova, M., Buonomo, S.B., Michaelis, C., Nairz, K., and Nasmyth, K. (1999). A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. Cell *98*, 91–103.

Krawchuk, M.D., DeVeaux, L.C., and Wahls, W.P. (1999). Meiotic chromosome dynamics dependent upon the rec8(+), rec10(+) and rec11(+) genes of the fission yeast Schizosaccharomyces pombe. Genetics *153*, 57–68.

Loidl, J., Klein, F., and Scherthan, H. (1994). Homologous pairing is reduced but not abolished in asynaptic mutants of yeast. J. Cell Biol. *125*, 1191–1200.

Loidl, J., Klein, F., and Engebrecht, J. (1998). Genetic and Morphological approaches for the Analysis of Meiotic Chromosomes in Yeast. 257–285. Academic Press.

Losada, A., Hirano, M., and Hirano, T. (1998). Identification of Xenopus SMC protein complexes required for sister chromatid cohesion. Genes Dev. *12*, 1986–1997.

Maguire, M.P. (1974). Letter: the need for a chiasma binder. J. Theor. Biol. *48*, 485–487.

Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell *91*, 35–45.

Molnar, M., Bahler, J., Sipiczki, M., and Kohli, J. (1995). The rec8 gene of Schizosaccharomyces pombe is involved in linear element formation, chromosome pairing and sister-chromatid cohesion during meiosis. Genetics *141*, 61–73.

Moore, D.P., and Orr-Weaver, T.L. (1998). Meiotic chromosome segregation. In Current Topics in Developmental Biology, Vol. 37. (Academic Press), pp. 263–299.

Nairz, K., and Klein, F. (1997). mre11S—a yeast mutation that blocks double-strand-break processing and permits nonhomologous synapsis in meiosis. Genes Dev. *11*, 2272–2290.

Nasmyth, K. (1999). Separating sister chromatids. Trends Biochem. Sci. *24*, 98–104.

Nasmyth, K., Peters, J.M., and Uhlmann, F. (2000). Splitting the chromosome: cutting the ties that bind sister chromatids. Science *288*, 1379–1385.

Nicklas, R.B. (1977). Chromosome distribution: experiments on cell hybrids and in vitro. Phil. Trans. R. Soc. Lond. *277*, 267–276.

Nicklas, R.B. (1988). The forces that move chromosomes in mitosis. Annu. Rev. Biophys. Chem. *17*, 431–449.

Orr-Weaver, T.L. (1999). The ties that bind: localization of the sister-chromatid cohesin complex on yeast chromosomes. Cell *99*, 1–4.

Parisi, S., McKay, M.J., Molnar, M., Thompson, M.A., van der Spek, P.J., van Drunen-Schoenmaker, E., Kanaar, R., Lehmann, E., Hoeijmakers, J.H., and Kohli, J. (1999). Rec8, a meiotic recombination and sister chromatid cohesion phosphoprotein of the Rad21p family conserved from fission yeast to humans. Mol. Cell. Biol. *19*, 3515–3528.

Piatti, S., Bohm, T., Cocker, J.H., Diffley, J.F., and Nasmyth, K. (1996). Activation of S-phase-promoting CDKs in late G1 defines a "point of no return" after which Cdc6 synthesis cannot promote DNA replication in yeast. Genes Dev. *10*, 1516–1531.

Ponticelli, A.S., and Smith, G.R. (1989). Meiotic recombination-deficient mutants of Schizosaccharomyces pombe. Genetics *123*, 45–54.

Pringle, J.R., Adams, A.E., Drubin, D.G., and Haarer, B.K. (1991). Immunofluorescence methods for yeast. Methods Enzymol. *194*, 565–602.

Rieder, C.L., and Cole, R. (1999). Chromatid cohesion during mitosis: lessons from meiosis. J. Cell Sci. *112*, 2607–2613.

Rieder, C.L., and Palazzo, R.E. (1992). Colcemid and the mitotic cycle. J. Cell Sci. *102*, 387–392.

Rieder, C.L., and Salmon, E.D. (1998). The vertebrate cell kinetochore and its roles during mitosis. Trends Cell Biol. *8*, 310–318.

Rockmill, B., Engebrecht, J.A., Scherthan, H., Loidl, J., and Roeder, G.S. (1995). The yeast MER2 gene is required for chromosome synapsis and the initiation of meiotic recombination. Genetics *141*, 49–59.

Salah, S., and Nasmyth, K. (2000). Destruction of the securin Pds1 occurs at the onset of anaphase during both meiotic divisions in yeast. Chromosoma *109*, 27–34.

Sambrook, J., Fritsch., E. F., and Maniatis, T. (1989). Molecular Cloning, Vol. 3. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics *122*, 19–27.

Skibbens, R.V., Corson, L.B., Koshland, D., and Hieter, P. (1999). Ctf7p is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery. Genes Dev. *13*, 307–319.

Storlazzi, A., Xu, L., Cao, L., and Kleckner, N. (1995). Crossover and noncrossover recombination during meiosis: timing and pathway relationships. Proc. Natl. Acad. Sci. USA 92, 8512–8516.

Sym, M., Engebrecht, J.A., and Roeder, G.S. (1993). ZIP1 is a synaptonemal complex protein required for meiotic chromosome synapsis. Cell *72*, 365–378.

Thierry, A., Gaillon, L., Galibert, F., and Dujon, B. (1995). Construction of a complete genomic library of Saccharomyces cerevisiae and physical mapping of chromosome XI at 3.7 kb resolution. Yeast *11*, 121–135.

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleiffer, A., and Nasmyth, K. (1999). Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. Genes Dev. *13*, 320–333.

Uhlmann, F., and Nasmyth, K. (1998). Cohesion between sister chromatids must be established during DNA replication. Curr. Biol. *8*, 1095–1101.

Uhlmann, F., Lottspeich, F., and Nasmyth, K. (1999). Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. Nature *400*, 37–42.

Uhlmann, F., Wernic, D., Poupart, M.-A., Koonin, E.V., and Nasmyth, K. (2000). Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast. Cell *103*, this issue, 375–386.

Varshavsky, A. (1997). The N-end rule pathway of protein degradation. Genes Cells 2, 13–28.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast *10*, 1793–1808.

Waizenegger, I.C., Hauf, S., Meinke, A., and Peters, J.-M. (2000). Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. Cell *103*, this issue, 399–410.

Watanabe, Y., and Nurse, P. (1999). Cohesin Rec8 is required for reductional chromosome segregation at meiosis. Nature *400*, 461–464.

Weiner, B.M., and Kleckner, N. (1994). Chromosome pairing via multiple interstitial interactions before and during meiosis in yeast. Cell *77*, 977–991.

Xu, L., and Kleckner, N. (1995). Sequence non-specific double-strand breaks and interhomolog interactions prior to double-strand break formation at a meiotic recombination hot spot in yeast. EMBO J. *14*, 5115–5128.

Zickler, D., and Kleckner, N. (1998). The leptotene-zygotene transition of meiosis. Annu. Rev. Genet. *32*, 619–697.

Spec SE mass spectrometer. Single-segment PSD spectra were recorded on isolated peptide $[M+H]^+$ ions. Phosphopeptides were sequenced (*18*) by nanoelectrospray MS/MS on a Micromass Q-ToF. Precursor ion spectra for *m/z* 79 (*14, 18*) were recorded on a modified Sciex API III+ triple quadrupole mass spectrometer equipped with a nanospray source.

32. J. L. Joyal *et al.*, *J. Biol. Chem.* **272**, 15419 (1997).
33. R. Verma *et al.*, *Science* **278**, 455 (1997).
34. K. Biemann, *Methods Enzymol.* **193**, 886 (1990).
35. Immortalized *Xenopus* melanophores were cultured as described (*36*). For in vivo binding and release experiments, eight 100-mm plates of melanophores per treatment were each transfected by electroporation with 10 μg of pcDNA3-myc-MGT or Ser^{1650} mutants of this construct. Transiently transfected cells were allowed to express protein for 48 hours before harvest.
36. S. L. Rogers *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3720 (1997).
37. This work was supported by NIH grant GM-52111 and NSF grant MCB95-13388. We thank R. Larson and A. Murray for discussions; R. Deshaies for Plx1; and J. Hammer (NHLB, NIH) for pCMV2-FLAG-MCST.

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Cohesin Cleavage by Separase Required for Anaphase and Cytokinesis in Human Cells

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Cell division depends on the separation of sister chromatids in anaphase. In yeast, sister separation is initiated by cleavage of cohesin by the protease separase. In vertebrates, most cohesin is removed from chromosome arms by a cleavage-independent mechanism. Only residual amounts of cohesin are cleaved at the onset of anaphase, coinciding with its disappearance from centromeres. We have identified two separase cleavage sites in the human cohesin subunit SCC1 and have conditionally expressed noncleavable SCC1 mutants in human cells. Our results indicate that cohesin cleavage by separase is essential for sister chromatid separation and for the completion of cytokinesis.

In eukaryotes, replicated DNA molecules remain attached to each other until the onset of anaphase. This sister chromatid cohesion depends on a protein complex called cohesin (*1*). In yeast, sister chromatid separation is initiated by cleavage of cohesin's subunit Scc1p/Mcd1p by the protease separase (*2, 3*). This reaction removes cohesin from chromosomes and may directly dissolve cohesion between sister chromatids. In metaphase, separase is activated by the anaphase-promoting complex or cyclosome (APC), which mediates the ubiquitin-dependent proteolysis of the separase inhibitor securin (*4–9*).

In vertebrates, cohesin is removed from chromosomes in two steps. During prophase and prometaphase, the bulk of cohesin dissociates from the arms of condensing chromosomes (*10*) by a mechanism that depends neither on the APC-separase pathway nor on cleavage of the human ortholog of Scc1p/Mcd1p, SCC1 (*11*). A small amount of cohesin remains in centromeric regions until metaphase and is removed from chromosomes only at the onset of anaphase (*9*). In spread chromosomes from HeLa cells arrested in a preanaphase state, the absence of SCC1 staining on chromatid arms correlates with the lack of arm cohesion (Fig. 1, A and B), supporting the notion that loss of cohesin is required for sister chromatid separation. The disappearance of residual amounts of SCC1 staining from centromeres coincides with the APC- and separase-dependent cleavage of a small amount of SCC1 (*9*). It is unknown, however, if this cleavage reaction, which affects maximally 10% of the total cellular cohesin, is required for anaphase.

To analyze the role of SCC1 cleavage in human cells, we identified two cleavage sites in SCC1. To map the NH_2-terminal site, we generated a series of in vitro–translated NH_2- and COOH-terminal truncation mutants of SCC1 and compared their electrophoretic mobilities to those of the COOH- and NH_2-terminal in vivo cleavage products, respectively (Fig. 1C). These analyses suggested that amino acid residues 168 to 182 contain the NH_2-terminal cleavage site. Comparison of this region with the recognition site consensus of yeast separase (*2, 12, 13*) suggested that human SCC1 is cleaved after Arg^{172} (Fig. 1D). The analysis of recombinant versions of SCC1 containing small deletions or point mutations in this region confirmed this hypothesis (Fig. 1E). The same strategy was used to identify Arg^{450} as the COOH-terminal SCC1 cleavage site (Fig. 1, D and E) (*14*). Mutation of both sites abolished SCC1 cleavage by separase in vitro (Fig. 1F). Comparison of these sites with known separase cleavage sites in budding and fission yeast (*2, 12, 13*) yields glutamate-X-X-arginine as the consensus for the sequence preceding the scissile peptide bond (Fig. 1D). In yeast, Cdc5p kinase mediates phosphorylation of serine residues at the P6 positions preceding the cleavage sites in Scc1p/Mcd1p, which enhances cleavage (*15*). In the NH_2-terminal cleavage site of human SCC1, this position is occupied by an aspartate residue, suggesting that separase prefers acidic residues at the P6 position.

We generated stable human HeLa cell lines in which physiologic amounts of wild-type or cleavage-site mutants of myc-tagged SCC1 are expressed upon addition of doxycycline (Fig. 2A) (*16*). Cell lines expressing noncleavable SCC1 proliferated more slowly than did lines expressing the wild-type or single-site mutants (*14*). Transgene expression was rapidly lost after expression of noncleavable SCC1 (Fig. 2B), indicating that the modified SCC1 interferes with proliferation. All SCC1 mutants were incorporated into 14*S* cohesin complexes (Fig. 2C) (*14*) and showed nuclear localization in interphase (Fig. 3H), implying that they acted as functional cohesin subunits. Fluorescence microscopy of mitotic cells (Fig. 2D) and incubation of chromatin fractions in meiotic *Xenopus* egg extracts (Fig. 2E) in which the APC-separase pathway is inactive (*11, 17*) suggested that the bulk of all SCC1 mutants can be dissociated from chromosomes by the prophase pathway, further demonstrating that this pathway does not depend on SCC1 cleavage. Analysis of SCC1 cleavage in mitotic *Xenopus* extracts using chromatin from the different HeLa cell lines as substrate confirmed that the different SCC1 mutants are noncleavable at either the NH_2- or the COOH-terminal site or at both (Fig. 2F).

Immunofluorescence microscopy suggested that most cells expressing wild-type SCC1 completed mitosis and cytokinesis normally (Fig. 3A), whereas multiple mitotic abnormalities were seen in cells expressing noncleavable SCC1 (Fig. 3B). In the latter cells, the cleavage furrow had often begun to ingress although anaphase had not occurred; i.e., the chromosomes remained at the spindle equator. In these cases, the cleavage furrow appeared to constrict the chromosome mass randomly in either a symmetric or an asymmetric manner (Fig. 3B), resembling the cut phenotype in fission yeast mutants defective in anaphase (*18*). No cyclin B staining could be observed in the human cells attempting

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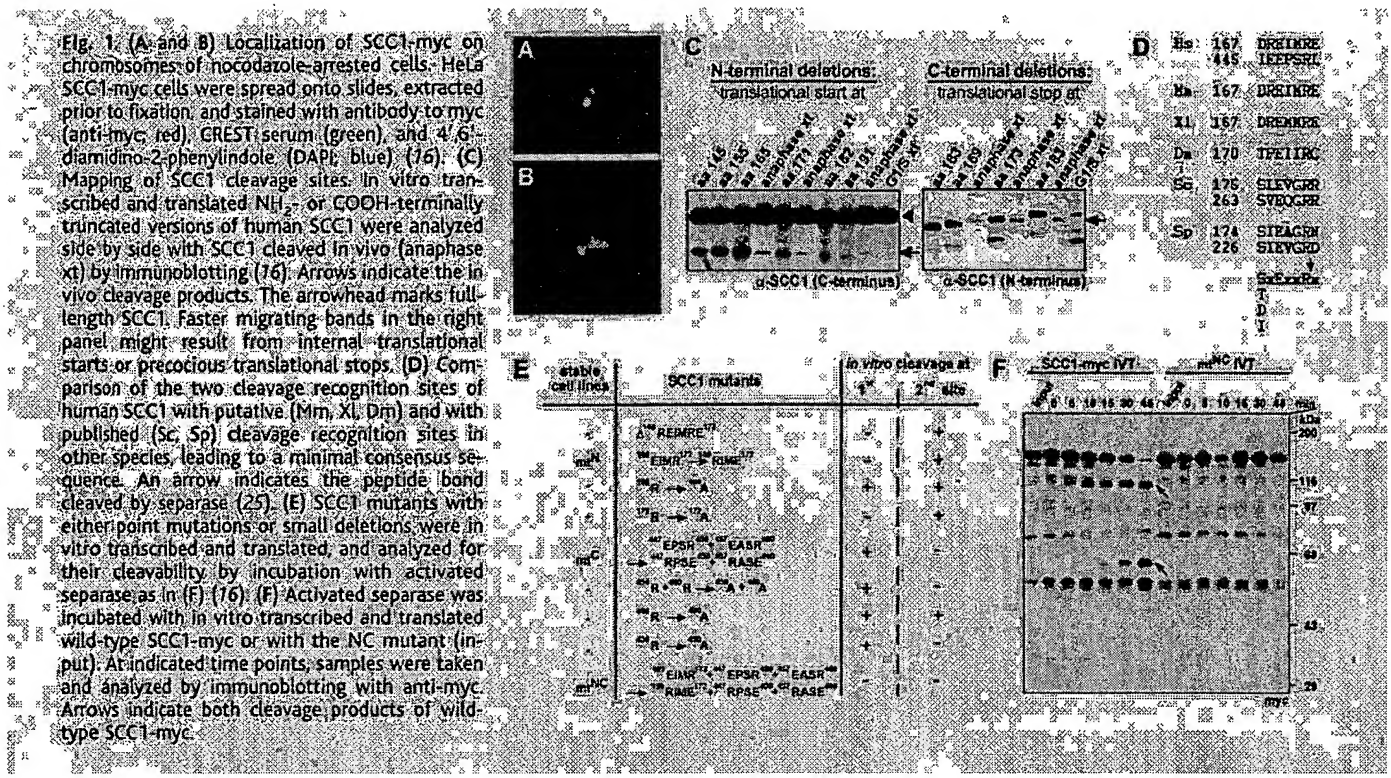

Fig. 1. (A and B) Localization of SCC1-myc on chromosomes of nocodazole-arrested cells. HeLa SCC1-myc cells were spread onto slides, extracted prior to fixation, and stained with antibody to myc (anti-myc, red), CREST serum (green), and 4′,6′-diamidino-2-phenylindole (DAPI, blue) (*16*). (C) Mapping of SCC1 cleavage sites. In vitro transcribed and translated NH_2- or COOH-terminally truncated versions of human SCC1 were analyzed side by side with SCC1 cleaved in vivo (anaphase xt) by immunoblotting (*16*). Arrows indicate the in vivo cleavage products. The arrowhead marks full-length SCC1. Faster migrating bands in the right panel might result from internal translational starts or precocious translational stops. (D) Comparison of the two cleavage recognition sites of human SCC1 with putative (Mm, Xl, Dm) and with published (Sc, Sp) cleavage recognition sites in other species, leading to a minimal consensus sequence. An arrow indicates the peptide bond cleaved by separase (*25*). (E) SCC1 mutants with either point mutations or small deletions were in vitro transcribed and translated, and analyzed for their cleavability by incubation with activated separase as in (F) (*16*). (F) Activated separase was incubated with in vitro transcribed and translated wild-type SCC1-myc or with the NC mutant (input). At indicated time points, samples were taken and analyzed by immunoblotting with anti-myc. Arrows indicate both cleavage products of wild-type SCC1-myc.

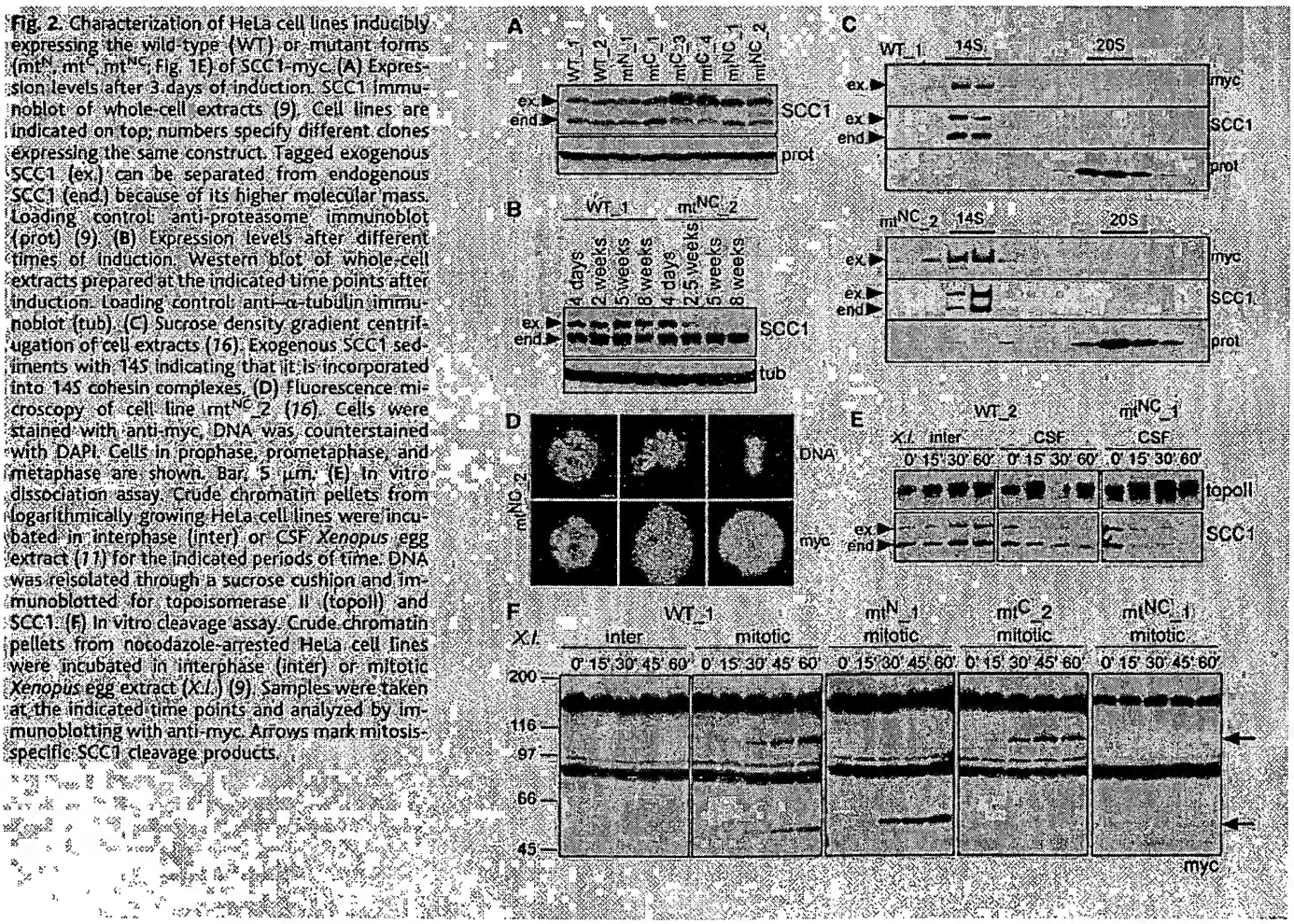

Fig. 2. Characterization of HeLa cell lines inducibly expressing the wild-type (WT) or mutant forms (mt^N, mt^C, mt^{NC}; Fig. 1E) of SCC1-myc. (A) Expression levels after 3 days of induction. SCC1 immunoblot of whole-cell extracts (*9*). Cell lines are indicated on top; numbers specify different clones expressing the same construct. Tagged exogenous SCC1 (ex.) can be separated from endogenous SCC1 (end.) because of its higher molecular mass. Loading control: anti-proteasome immunoblot (prot) (*9*). (B) Expression levels after different times of induction. Western blot of whole-cell extracts prepared at the indicated time points after induction. Loading control: anti–α-tubulin immunoblot (tub). (C) Sucrose density gradient centrifugation of cell extracts (*16*). Exogenous SCC1 sediments with 14S indicating that it is incorporated into 14S cohesin complexes. (D) Fluorescence microscopy of cell line mt^{NC}_2 (*16*). Cells were stained with anti-myc, DNA was counterstained with DAPI. Cells in prophase, prometaphase, and metaphase are shown. Bar, 5 μm. (E) In vitro dissociation assay. Crude chromatin pellets from logarithmically growing HeLa cell lines were incubated in interphase (inter) or CSF *Xenopus* egg extract (*11*) for the indicated periods of time. DNA was reisolated through a sucrose cushion and immunoblotted for topoisomerase II (topoII) and SCC1. (F) In vitro cleavage assay. Crude chromatin pellets from nocodazole-arrested HeLa cell lines were incubated in interphase (inter) or mitotic *Xenopus* egg extract (*X.l.*) (*9*). Samples were taken at the indicated time points and analyzed by immunoblotting with anti-myc. Arrows mark mitosis-specific SCC1 cleavage products.

cytokinesis in the absence of anaphase (*14*), implying that the APC and therefore presumably also separase had been activated. The Aurora-B kinase relocates from centromeres to the spindle midzone around the same time as SCC1 is cleaved (Fig. 3C) (*19*). Aurora-B could also be detected at the spindle midzone in cells that failed to separate their chromatids due to uncleavable SCC1 (Fig. 3, D and E), suggesting that Aurora-B relocation does not depend on chromatid separation. In addition, anaphase bridges were frequently observed in cells expressing noncleavable SCC1, but a significant fraction of cells also appeared to undergo anaphase normally (Fig. 3F). It is possible that cells undergoing a normal anaphase expressed less noncleavable SCC1 than did cells showing anaphase defects. Inspection of cells expressing SCC1 mutants that can only be cleaved at either one of the two cleavage sites revealed an increase in the frequency of cells with anaphase bridges, but no cells with the cut phenotype could be observed (Fig. 3F).

In interphase cells expressing noncleavable SCC1, micronuclei, enlarged nuclei, and multiple nuclei were frequently observed (Fig. 3G). Fluorescence-activated cell sorting (FACS) (*16*) revealed that expression of noncleavable SCC1 coincided with a pronounced increase in aneuploid cells, representing, in some samples, 30% of all transgene-expressing cells (Fig. 3H). To analyze how cells with these phenotypes arise, we analyzed cells expressing SCC1 mutants and a green fluorescent protein (GFP)–tagged version of histone H2B (*20*) by video microscopy (*16*). Expression of wild-type SCC1 did not interfere with mitotic progression, whereas cells expressing noncleavable SCC1 initiated cytokinesis without segregating chromosomes (Fig. 4, A and B) (*16*). The latter cells stretched chromosomes toward the opposite poles of the cleaving cell and partially constricted the chromosomes by the ingressing cleavage furrow, causing the cut phenotype seen in fixed cells (Fig. 3B). Eventually, the cleavage furrow regressed, and the cells entered interphase without completing cytokinesis, often containing an enlarged and irregularly shaped nucleus (Fig. 4B).

To examine the karyotype of these cells, we induced expression of noncleavable SCC1 for 3 days to allow on average one round of mitosis in the presence of noncleavable cohesin, then added colchicine to accumulate cells in the subsequent mitosis and analyzed chromosome spreads by microscopy (*16*). Many spreads contained diplochromosomes in which two pairs of sister chromatids were aligned in parallel, reminiscent of the chromosomes observed in *Drosophila* mutants defective in anaphase (*21*). Such karyotypes were infrequent in spreads from cells expressing wild-type SCC1 or single-site cleavage SCC1 mutants (Fig. 4C) (*14*). These observations suggest that cells expressing noncleavable SCC1 reenter interphase with sis-

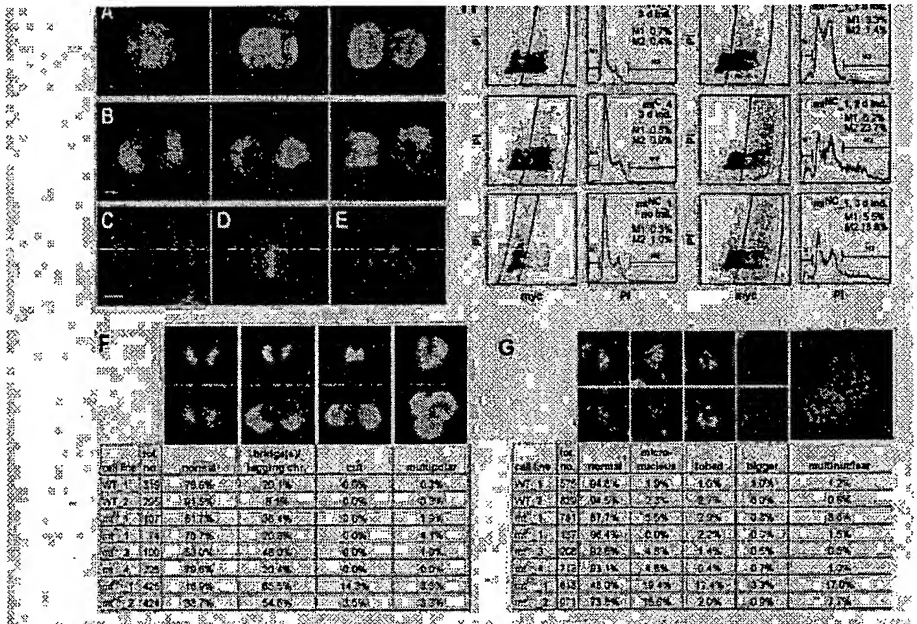

Fig. 3. Phenotype of HeLa cells expressing noncleavable SCC1-myc. (A) Normal mitoses in cells that expressed SCC1-myc WT (two left panels) or SCC1-myc mt^{C} (right panel). Cells were stained with anti-myc (green) and DAPI (blue) (*16*). Bar, 5 μm. (B) Abnormal mitoses in cells that expressed hSCC1-myc mt^{NC}. Same staining as in (A). Bar, 5 μm. (C through E) Cells expressing hSCC1-myc WT (C) or hSCC1-myc mt^{NC} (D and E) were extracted prior to fixation and costained for Aurora-B (green) and hSCC1-myc (red). DNA was stained with DAPI (blue). Bar, 5 μm. (F) Morphology of cells undergoing cytokinesis determined 3 days after induction. Cells undergoing cytokinesis and expressing wild-type or mutant versions of SCC1-myc were classified as follows: (normal) normal anaphase or telophase, [bridge(s)/lagging chr.] cells in anaphase or telophase that showed either chromatin bridges or lagging chromosomes, (cut) cells undergoing cytokinesis without having separated their chromatin, (multipolar) multipolar anaphases or telophases. The total number (tot. no.) of cells counted for each cell line is indicated. Upper panel: DNA stained with DAPI. Lower panel: DNA in blue, SCC1-myc in green. Bar, 5 μm. (G) Morphology of interphase nuclei determined 5 to 7 days after induction. The total number (tot. no.) of cells counted for each cell line is indicated. Upper panel: DNA stained with DAPI. Lower panel: DNA in blue, α-tubulin in red, and SCC1-myc in green. Bar, 10 μm. (H) FACS analysis of DNA content. Cells were stained for SCC1-myc (myc) and DNA (propidiumiodide, PI). Only cells gated for in the dot plot are shown in the histogram panel. The percentage of hypodiploid (M1) or hyperdiploid (M2) cells is indicated.

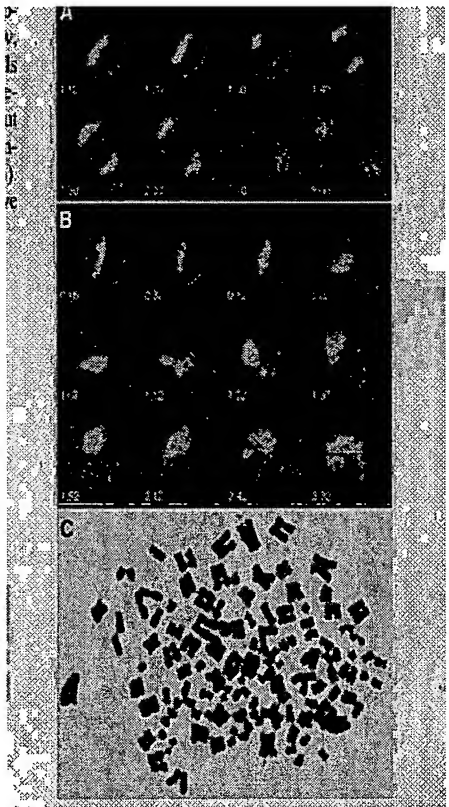

Fig. 4. Cells exhibiting a "cut" phenotype eventually fail to undergo cytokinesis, and diplochromosomes are present after 3 days of induction. Video microscopy of cells expressing wild-type (A) or noncleavable (B) SCC1-myc (*16*). Cell lines WT_1 and mt^{NC}_1 were stably transfected with H2B-GFP (*20*) to visualize DNA. Cells were induced to express SCC1-myc for 2 days before analysis. Selected pictures taken at the indicated time points (hours:min) are shown (representative of three recordings). Nomarski is shown in red, overlayed by H2B-GFP in green. (C) Chromosome spread of cells expressing noncleavable SCC1-myc. Cell line mt^{NC}_1 was induced to express SCC1-myc for 3 days. Mitotic cells were harvested after 2 hours treatment with colchicine, incubated in hypotonic solution, fixed, and spread. Coverslips were stained with Giemsa. Of the spreads in this preparation, 5.4% showed several diplochromosomes (compared to none in cell line WT_2).

ter chromatids still connected. These cells nevertheless re-replicate their DNA, resulting in the formation of diplochromosomes in the subsequent mitosis (Fig. 4C). Repeated cycles of abortive mitosis and DNA re-replication may cause formation of the polyploid or multinucleate cells observed by FACS and microscopy (Fig. 3). Paradoxically, spread diplochromosomes typically appeared as pairs of chromosomes whose centromeric regions were not connected (Fig. 4C). Their nonrandom parallel orientation on the coverslips can only be explained, however, by assuming that all four chromatids had been connected by cohesion prior to the hypotonic spreading procedure.

Our results suggest that cohesin cleavage is essential for sister chromatid separation and cytokinesis, even though only a minor amount of SCC1 is cleaved in mitosis (*9*). It is therefore likely that the anaphase defects of cells in which separase activity is compromised by securin overexpression or deletion are similarly caused by defects in SCC1 cleavage (*22, 23*). Because SCC1 cleavage coincides with the disappearance of cohesin from centromeres (*9*), SCC1 cleavage may preferentially dissolve cohesion at centromeres. It is possible that the cytokinesis defects induced by noncleavable cohesin are caused by a mechanical inability of the cleavage furrow to cut through chromosomes remaining in the furrow plane. Alternatively, it is conceivable that a surveillance mechanism prevents cytokinesis in the presence of unseparated sister chromatids.

Our observations also support the notion that sister separation is essential neither for cyclin B destruction and exit from mitosis nor for DNA re-replication in the next cell cycle (*2, 4–8, 21*) — and is also not necessary for relocalization of Aurora-B from centromeres to the spindle midzone. Naturally occurring defects in separase activation and SCC1 cleavage would therefore not simply block cell cycle progression, but could instead cause chromosome nondisjunction events that could subsequently cause continued chromosomal instability by initiating chromosome breakage-fusion-bridge cycles (*24*). It will therefore be of interest to determine if defects in separase regulation contribute to the genomic instability observed in human tumors.

References and Notes

1. K. Nasmyth, J. M. Peters, F. Uhlmann, *Science* **288**, 1379 (2000).
2. F. Uhlmann, F. Lottspeich, K. Nasmyth, *Nature* **400**, 37 (1999).
3. F. Uhlmann, D. Wernic, M. A. Poupart, E. V. Koonin, K. Nasmyth, *Cell* **103**, 375 (2000).
4. O. Cohen-Fix, J. M. Peters, M. W. Kirschner, D. Koshland, *Genes Dev.* **10**, 3081 (1996).
5. H. Funabiki *et al.*, *Nature* **381**, 438 (1996).
6. R. Closk *et al.*, *Cell* **93**, 1067 (1998).
7. H. Zou, T. J. McGarry, T. Bernal, M. W. Kirschner, *Science* **285**, 418 (1999).
8. O. Lelsmann, A. Herzig, S. Heidmann, C. F. Lehner, *Genes Dev.* **14**, 2192 (2000).
9. I. C. Waizenegger, S. Hauf, A. Meinke, J. M. Peters, *Cell* **103**, 399 (2000).
10. A. Losada, M. Hirano, T. Hirano, *Genes Dev.* **12**, 1986 (1998).
11. I. Sumara, E. Vorlaufer, C. Gieffers, B. H. Peters, J. M. Peters, *J. Cell Biol.* **151**, 749 (2000).
12. S. B. Buonomo *et al.*, *Cell* **103**, 387 (2000).
13. T. Tomonaga *et al.*, *Genes Dev.* **14**, 2757 (2000).
14. S. Hauf, I. C. Waizenegger, J.-M. Peters, data not shown.
15. G. Alexandru, F. Uhlmann, K. Mechtler, M. Poupart, K. Nasmyth, *Cell* **105**, 459 (2001).
16. Supplementary material is available at Science Online at www.sciencemag.org/cgi/content/full/293/5533/1320/DC1.
17. E. Vorlaufer, J. M. Peters, *Mol. Biol. Cell* **9**, 1817 (1998).
18. M. Yanagida, *Trends Cell Biol.* **8**, 144 (1998).
19. E. A. Nigg, *Nature Rev. Mol. Cell Biol.* **2**, 21 (2001)
20. T. Kanda, K. F. Sullivan, G. M. Wahl, *Curr. Biol.* **8**, 377 (1998).
21. R. Stratmann, C. F. Lehner, *Cell* **84**, 25 (1996).
22. A. Zur, M. Brandeis, *EMBO J.* **20**, 792 (2001).
23. P. V. Jallepalli *et al.*, *Cell* **105**, 445 (2001).
24. B. McClintock, *Cold Spring Harbor Symp. Quant. Biol.* **9**, 72 (1941).
25. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. X indicates any residue.

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Vascular Abnormalities and Deregulation of *VEGF* in *Lkb1*-Deficient Mice

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The *LKB1* tumor suppressor gene, mutated in Peutz-Jeghers syndrome, encodes a serine/threonine kinase of unknown function. Here we show that mice with a targeted disruption of *Lkb1* die at midgestation, with the embryos showing neural tube defects, mesenchymal cell death, and vascular abnormalities. Extraembryonic development was also severely affected; the mutant placentas exhibited defective labyrinth layer development and the fetal vessels failed to invade the placenta. These phenotypes were associated with tissue-specific deregulation of vascular endothelial growth factor (*VEGF*) expression, including a marked increase in the amount of *VEGF* messenger RNA. Moreover, VEGF production in cultured *Lkb1*$^{-/-}$ fibroblasts was elevated in both normoxic and hypoxic conditions. These findings place *Lkb1* in the VEGF signaling pathway and suggest that the vascular defects accompanying Lkb1 loss are mediated at least in part by VEGF.

Germ line mutations of the *LKB1* gene cause Peutz-Jeghers syndrome, which is characterized by gastrointestinal polyposis, abnormal melanin pigmentation, and increased risk of cancer (*1*). The *Lkb1* gene encodes a serine/threonine kinase of unknown function with no identified in vivo substrates and a ubiquitous expression pattern during mouse development (*2*). To study the function of *Lkb1*, we generated *Lkb1*-deficient mice.

Two independent gene-targeting strategies were used to functionally disrupt *Lkb1* in the murine germ line (*3, 4*). In *Lkb1* heterozygous (*Lkb1*$^{+/-}$) intercrosses, both *Lkb1*$^{+/+}$ (n = 87) and *Lkb1*$^{+/-}$ (n = 177) animals were observed at expected frequencies, whereas no *Lkb1*$^{-/-}$ animals were obtained. Analysis of *Lkb1*$^{-/-}$ embryos throughout embryonic development revealed no abnormalities before embryonic day 7.5 (E7.5), and most embryos appeared to develop normally up to E8.0. Macroscopic analysis of *Lkb1*$^{-/-}$ embryos beyond E8.25 revealed multiple abnormalities, including a failure of the embryo to turn, a defect in neural tube closure, and a hypoplastic or absent first branchial arch (Fig. 1A). No viable embryos were recovered after E11.0, indicating that Lkb1 is essential for embryonic development.

Whole-mount in situ hybridization was used to study the integrity of various developmental

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Example 14: Product by Function

Specification: The specification exemplifies a protein isolated from liver that catalyzes the reaction of A → B. The isolated protein was sequenced and was determined to have the sequence as set forth in SEQ ID NO: 3. The specification also contemplates but does not exemplify variants of the protein wherein the variant can have any or all of the following: substitutions, deletions, insertions and additions. The specification indicates that procedures for making proteins with substitutions, deletions, insertions and additions is routine in the art and provides an assay for detecting the catalytic activity of the protein.

Claim:

A protein having SEQ ID NO: 3 and variants thereof that are at least 95% identical to SEQ ID NO: 3 and catalyze the reaction of A → B.

Analysis:

A review of the full content of the specification indicates that a protein having SEQ ID NO: 3 or variants having 95% identity to SEQ ID NO: 3 and having catalytic activity are essential to the operation of the claimed invention. The procedures for making variants of SEQ ID NO: 3 are conventional in the art and an assay is described which will identify other proteins having the claimed catalytic activity. Moreover, procedures for making variants of SEQ ID NO: 3 which have 95% identity to SEQ ID NO: 3 and retain its activity are conventional in the art.

A review of the claim indicates that variants of SEQ ID NO: 3 include but are not limited to those variants of SEQ ID NO: 3 with substitutions, deletions, insertions and additions; but all variants must possess the specified catalytic activity and must have at least 95% identity to the SEQ ID NO: 3. Additionally, the claim is drawn to a protein which **comprises** SEQ ID NO: 3 or a variant thereof that has 95% identity to SEQ ID NO: 3. In other words, the protein claimed may be larger than SEQ ID NO: 3 or its variant with 95% identity to SEQ ID NO: 3. It should be noted that "having" is open language, equivalent to "comprising".

The claim has two different generic embodiments, the first being a protein which comprises SEQ ID NO: 3 and the second being variants of SEQ ID NO: 3. There is a single species disclosed, that species being SEQ ID NO: 3.

A search of the prior art indicates that SEQ ID NO: 3 is novel and unobvious.

There is actual reduction to practice of the single disclosed species. The specification indicates that the genus of proteins that must be variants of SEQ ID NO: 3 does not have substantial variation since all of the variants must possess the specified catalytic activity and must have at least 95% identity to the reference sequence, SEQ ID NO: 3. The single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity. One of skill in the art would conclude that

applicant was in possession of the necessary common attributes possessed by the members of the genus.

Conclusion: The disclosure meets the requirements of 35 USC §112 first paragraph as providing adequate written description for the claimed invention.